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Role and Regulation of Fat Specific Protein (FSP27) in Lipolysis in 3T3-L1 Adipocytes: A Dissertation

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ROLE AND REGULATION OF FAT SPECIFIC PROTEIN (FSP27) IN LIPOLYSIS IN 3T3-L1 ADIPOCYTES

A Dissertation Presented

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Dedicated to

Avani, Bayabya, and Sajja

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Abstract

The alarming rate of increase in incidence and prevalence of the type 2 diabetes mellitus has prompted intense research on understanding the pathogenesis of the type 2 diabetes. It is observed that the development of type 2 diabetes is preceded by a state of insulin resistance and obesity. Previous studies have suggested that the obesity induced insulin resistance may be mediated by elevated levels of circulating free fatty acids (FFAs). The increase in circulating levels of FFAs may be contributed by the release of FFAs from stored triglycerides (TG) in adipocytes via lipolysis. It is hypothesized that the decrease in levels of circulating FFAs by sequestration and storage of FFAs in adipocytes may prevent deleterious effects of FFAs on insulin sensitivity. Recently our lab and others have shown that the storage of TG in adipocytes is promoted by a novel protein, Fat Specific Protein 27 (FSP27). Although, these studies also revealed FSP27 to be a lipid droplet associated protein that suppresses lipolysis to enhance TG accumulation in adipocytes, the role of FSP27 in lipolysis remains largely undetermined. Therefore, this study investigates the role and regulation of FSP27 in adipocytes in both the basal state, as well as during lipolysis.

The studies presented here show FSP27 to be a remarkably short-lived protein (half-life=15 min) due to its rapid ubiquitination and proteasomal degradation. Thus, I tested the hypothesis that lipolytic agents like the

cytokine, TNF-α and the catecholamine isoproterenol modulate FSP27 protein levels to regulate FFA release. Consistent with this concept, TNF-α markedly decreased FSP27 mRNA and protein along with lipid droplet size as it increased lipolysis in cultured adipocytes**.** Similarly, FSP27 depletion using siRNA mimicked the effect of TNF-α to enhance lipolysis, while maintaining stable FSP27 protein levels by expression of HA epitope-tagged FSP27 blocked TNF-α mediated lipolysis. In contrast, the robust lipolytic action of isoproterenol is paradoxically associated with increases in FSP27 protein and a delayed degradation rate that corresponds to decreased ubiquitination. This catecholamine-mediated increase in FSP27 abundance, probably a feedback mechanism to restrain excessive lipolysis by catecholamines, is mimicked by forskolin or 8-Bromo-cAMP treatment, and prevented by Protein Kinase A (PKA) inhibitor KT5720 or PKA depletion using siRNA. These results show that isoproterenol stabililizes FSP27 via the canonical PKA pathway and increased cAMP levels. However, the work presented here also suggests that FSP27 does not get phosphorylated in response to isoproterenol treatment, and the stabilization of FSP27 is independent of isoproterenol mediated lipolysis.

The data presented in this thesis not only identifies the regulation of FSP27 as an important intermediate in mechanism of lipolysis in adipocytes in

response to TNF-α and isoproterenol, but also suggests that FSP27 may be a possible therapeutic target to modulate lipolysis in adipocytes.

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List of Frequently used Abbreviations

FSP27	Fat Specific Protein 27
CIDEC	Cell Death-Inducing DFFA-like Effector C
CIDE	Cell Death-Inducing DFFA-like Effector
TNFα	Tumor Necrosis Factor α
TG	Triacylglycerol
FFA	Free Fatty Acids
ATGL	Adipose Triacylglycerol Lipase
HSL	Hormone Sensitive Lipase
PAT	Perilipin-Adipophilin-TIP47;
PLIN	Perilipin
ADRP	Adipocyte Differentiation Related Protein
TIP47	Tail Interacting Protein 47;
OXPAT/ MLDP,	Oxidative tissues-enriched PAT protein/Myocardial lipid droplet protein;
PPARγ	Peroxisome Proliferator-Activated Receptor γ;
C/EBPα	CCAAT/Enhancer binding protein α;
PKA	cAMP dependent Protein Kinase A;
PKB	Protein Kinase B
PKC	Protein Kinase C
JNK	c-jun N-terminal Kinase

PI3K	Phosphoinositide 3-Kinase
MEK-ERK	Mitogen-activated Protein Kinase- Extracellular signal-related Kinase
GLUT	Glucose Transporter
IR	Insulin Receptor
IRS	Insulin Receptor substrate
PDE	Phosphodiesterase
MCP-1	monocyte chemoattractant protein-1
ACC	Acetyl-Coenzyme A Carboxylase
FAS	Fatty acid synthase
siRNA	small interfering RNA
WAT	White adipose tissue
BAT	Brown adipose tissue
Scr	Scrambled
ANOVA	Analysis of Variance.

Chapter I

INTRODUCTION

The scientific and technological advancement providing increased availability of food and a decreased need for physical work have shaped the present day human niche of overfeeding and under utilization of consumed calories. As a result, obesity and its subsequent result – Diabetes Mellitus are increasing at such alarming rates that both obesity and diabetes have become pandemic. According to the 2007 National Diabetes Fact Sheet (published on American Diabetes Association website, http://www.diabetes.org/diabetes-basics/diabetes-statistics/), 7.8% of the population in the United States has diabetes, and 1.6 million new cases of diabetes are diagnosed each year among people aged 20 yr and older. Even more disturbing is the trend that obesity and diabetes are becoming more prevalent in children and adolescence. It is estimated that 0.22% of the people in this age group have diabetes. In addition to the health problems, the management of diabetes imposes a huge economic burden. In 2007, the total cost of diagnosed diabetes was $174 billion in the United States alone. Therefore, obesity and diabetes are the nation's current major medical burdens.

Diabetes mellitus is a disorder of glucose homeostasis. The human body maintains the healthy serum glucose level of 4-7 mM through a balance between the input of glucose into the circulation from feeding, and the output

of glucose from the circulation by uptake and utilization by various tissues. Although, the maintenance of normal blood glucose is regulated by various hormones, the critical hormone required for glucose homeostasis is insulin. Any disturbance in the level of insulin or its function is the key to the development of diabetes. There is insulin deficiency in type I diabetes mellitus where as there is insulin resistance in type II diabetes mellitus. Both insulin deficiency as well as insulin resistance result in high glucose levels that become toxic to various tissue, thus giving rise to complications such as blindness, end-stage renal disease, and non-traumatic loss of limb (1).

The development of type II diabetes is related to a heritable genetic aspect as well as to the modifiable environmental factors such as diet and exercise. A study involving the Malmo Preventive Project and Botnia study in Finland consisting of 18,831 people showed the increased association of family history of diabetes in first-degree relatives, and genetic risk alleles to the development of diabetes. It also showed a higher incidence of diabetes in people when the genetic risk was combined with environmental factor such as obesity (2). The extensive study of the development of diabetes in obesity has suggested inflammation and FFAs as important mediators in obesity related diabetes, but the underlying mechanisms are yet to be fully understood. Unraveling these mechanisms will help to elucidate their role in the progression from obesity to diabetes. This understanding is important, as

it can provide a basis for newer and better therapies in the treatment and cure for diabetes.

Adipose Tissue: An endocrine organ

There are two different types of adipose tissues in humans and rodents: brown adipose tissue (BAT) and white adipose tissue (WAT). BAT, named because of its brown color, has a thermogenic function. BAT was previously thought to be present only in infants, however, recent evidences have shown that there are brown adipocytes interspersed in WAT in adult humans (3). WAT on the other hand, is a soft, yellowish tissue that forms a few large depots in the body mainly subcutaneous and visceral in location. But there are also smaller depots present in the heart, large blood vessels, lymph nodes, bone marrow, kidney, adrenal gland, and the brain (4). WAT is comprised mainly of adipocytes or fat cells, along with stromal vascular cells consisting of fibroblasts, stem cells, and inflammatory cells (5). This thesis focuses on WAT and white adipocytes herein referred to as adipocytes.

Adipogenesis

The differentiation of adipocytes from fibroblasts with adipogenic potential is a complex process known as adipogenesis. Adipogenesis is predominantly controlled by peroxisome proliferator-activated receptor γ (PPAR γ) that is now considered the master regulator of adipogenesis (6, 7). PPARγ is a

transcription factor that is activated by various endogenous ligands such as polyunsaturated fatty acids, prostaglandin J_2, as well as exogenous ligands such as ibuprofen, indomethacin, and chiefly thiazolidinediones (TZDs) (8-11). Although PPARγ is considered the most important transcription factor, the process of adipocyte differentiation starts with the expression of two other transcription factors: C/EBPβ and C/EBPδ, that induce the expression of PPARγ and C/EBPα. PPARγ and C/EBPα act as a positive regulator of each other, and the high expression of these transcription factors is detected in terminal differentiation of adipocytes (7, 12). PPARγ drives the expression of many adipocyte genes such as lipoprotein lipase (LPL) (13), phosphoenolpyruvate carboxykinase (PEPCK) (14), acyl-CoA synthetase (ACS) (15), perilipin (16), and Cell Death-Inducing DFFA-like Effector A (CIDEA) (17). These PPARγ target genes, especially the ones involved in lipogenesis and lipid storage, ultimately direct adipocytes towards storing FFAs as triglycerides in lipid droplets.

Endocrine Functions of Adipose tissue

It is well known that adipocytes in WAT store excess energy derived from ingested food in their lipid droplets (18), but with the groundbreaking discovery of Leptin in 1995 (19), the role of WAT as an endocrine organ has started to emerge. In addition to leptin, WAT also secretes other hormones such as adiponectin, resistin, omentin, chemerin, retinol binding protein 4,

vaspin, and visfatin. These hormones secreted by WAT are collectively called adipokines. Studies on adipokines have supported their role in regulation of glucose and lipid metabolism, inflammation, atherosclerosis, hemostasis, and blood pressure (5). Furthermore, lipodystrophy, a disorder of adipose tissue formation, is associated with the insulin resistance and type 2 diabetes mellitus (20-23) , thus reinforcing the importance of the endocrine functions of adipose tissue.

Adiponectin (Acrp30)

One of the important adipokines is adiponectin, a 30-kDa protein secreted from adipocytes from an intermediate stage of adipogenesis onwards (24, 25). A deficiency of adiponectin *in vivo* has been shown to lead to insulin resistance (26-28) whereas increased adiponectin in transgenic mice has been associated with insulin sensitivity (29) and suppression of endogenous glucose production (30). There is also an association between reduced plasma adiponectin with obesity and insulin resistance in mice suggesting a role of adiponectin in insulin sensitivity (25, 31). The role of adiponectin in regulating whole body insulin sensitivity is further supported by the studies that show administration of recombinant adiponectin to rodents increases glucose uptake and fat oxidation in muscle, and decreases glucose production from liver (32-34). In addition to the insulin sensitizing effects in adipocytes, muscle, and liver, the global effect of adiponectin could also be

due to increased insulin secretion from the pancreas (35), and to the modification of food intake and energy expenditure through its actions in central nervous system (CNS) (36).

Adiponectin secreted from adipocytes circulates in plasma as a trimer, hexamer or 12- to 18-mer, and each form is thought to have a different activity (37, 38). The effects of adiponectin are mediated through adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2), both ubiquitously expressed but AdipoR1 is expressed predominantly in skeletal muscle while AdipoR2 is expressed more in liver (39, 40). Research investigating the mechanism of adiponectin actions suggest that activation of adiponectin receptors activates AMP-activated protein kinase (AMPK), and increases expression of PPARα target genes such as acyl-coenzyme oxidase, and uncoupler protein (UCP2) to increase fatty acid oxidation and energy expenditure (41).

Consistent with the data in animal models, the levels of circulating adiponectin in humans is negatively correlated with the insulin resistance (42-44), and type 2 diabetes (42, 45). In addition to this, single nucleotide polymorphisms (SNPs) in adiponectin gene have been reported to be associated with insulin resistance and type 2 diabetes mellitus (46). TZDs (anti-diabetic, therapeutic agent) are also known to cause increases in the expression and secretion of adiponectin (47-49). Taken together, these data

indicate that adiponectin is an important adipokine with a role in insulin sensitivity.

Leptin

Leptin is another adipokine that has been shown to regulate food intake and energy expenditure. The deficiency of leptin has been reported to lead to obesity and insulin resistance in rodents as well as humans. The effects of leptin were first observed in 1950 when a random mutation in a gene produced obese mice (*ob/ob*) within a colony at Jackson Laboratory (50). The gene '*Ob*' itself was cloned only in 1994 (51), and the '*Ob*' gene product-Leptin and its effect on feeding and energy expenditure were discovered in 1995 (19).

Circulating leptin, a small 16 KDa protein, is able to cross the blood-brain-barrier to act on the arcuate nucleus, a region of the hypothalamus in the central nervous system. Prior evidences suggest that stimulation of the leptin receptor by leptin activates the JAK-STAT signaling pathway that regulates food intake and body weight, as well as PI3K signaling pathway that regulates glucose metabolism. Because, leptin stimulates two different pathways to regulate body weight and glucose metabolism, mice with the mutation in the leptin receptor (*db/db*) can't activate the JAK-STAT pathway but can still signal through the PI3K pathway. These *db/db* mice are obese and

Adipokine	Function
Adiponectin	↑Insulin sensitivity; ↑Insulin secretion from pancreas,
Leptin	↓ food intake; ↑Insulin sensitivity; prevents ectopic lipid accumulation in liver and muscle
Omentin	↑Insulin sensitivity
Chemerin	Conflicting results on insulin sensitivity, needs further investigation
Vaspin	↑Insulin sensitivity
Resistin	↑Insulin resistance; ↓ Insulin sensitivity
Retinol Binding Protein 4	↑Insulin resistance; ↓ Insulin sensitivity
Visfatin	↑Insulin resistance; ↓ Insulin sensitivity; conflicting results in humans
Inflammatory Cytokines (TNF-α, IL-6, MCP-1)	TNF-α & IL-6: ↑Insulin resistance; ↓ Insulin sensitivity; MCP-1: ↑ Macrophage infiltration in adipose tissue

Table 2.1: List of adipokines and their effect on insulin sensitivity

hyperphagic due to the defect in JAK-STAT pathway, but have only mild glucose intolerance that can be corrected with calorie restriction (5).

In addition to the actions of leptin via CNS, the insulin sensitizing effect of leptin may also mediated by prevention of ectopic lipid accumulation in liver and muscle (52, 53). Studies have also shown that leptin protects the pancreas from lipid accumulation caused by a high calorie diet and promotes its function, in addition to inhibiting insulin secretion in lean animals (54).

The secretion of leptin is observed to be proportional to the adipose tissue mass, and thus there are higher levels of leptin in obese individuals compared to the lean ones. However, chronic elevation of leptin has been shown to have attenuated effects on improving insulin sensitivity despite the presence of a functional leptin receptor, that indicates the development of leptin resistance in presence of persistent high levels of circulating leptin (55). Thus, the maintenance of leptin levels, as well as leptin receptor function is important to regulate food intake, body weight, and insulin sensitivity.

Omentin

Omentin is an adipokine that has not been well characterized but has been shown to increase insulin stimulated glucose transport and AKT phosphorylation in human subcutaneous and visceral adipocytes. It is

secreted by the visceral stromal vascular cells. The levels of omentin 1, the major isoform of omentin present in plasma, is found to negatively correlate with adiponectin and HDL levels (5). Although it has been postulated that omentin plays a role in enhancing insulin sensitivity, the mechanism of this adipokine has yet to be uncovered.

Chemerin

The most recently discovered adipokine is Chemerin. It is highly expressed in liver and white adipose tissue (56, 57) and is upregulated during adipogenesis (58). Previous studies in 3T3-L1 adipocytes have shown that chemerin increases insulin-stimulated glucose uptake and IRS1 phosphorylation, whereas studies in activated macrophages expressing chemerin receptor have shown that chemerin has an anti-inflammatory effect (5). Rodent and human studies have revealed conflicting results regarding the role of chemerin in diabetes. *Db/db* mice have been shown to have decreased chemerin in adipose tissue compared to the controls, whereas chemerin is increased in adipose tissue from glucose intolerant and diabetic sand rat (56, 59). In humans, chemerin did not correlate with type 2 diabetes but in normal glucose tolerant subjects, chemerin levels have been associated with BMI, triglycerides, and blood pressure (56). Due to these conflicting results, the role of chemerin in insulin sensitivity remains to be verified and explored further.

Vaspin

Vaspin or visceral adipose tissue-derived serine protease inhibitor is an adipokine identified in visceral adipose tissue of Otsuka Long-Evans Tokushima fatty rat (OLETF), a new non-insulin dependent diabetes mellitus rat model. Vaspin levels decrease with the increasing body weight and glucose intolerance, whereas administration of recombinant vaspin to diet-induced obese rodents improves glucose tolerance and insulin sensitivity suggesting a positive correlation of vaspin with insulin sensitivity (5). Although, similar insulin sensitivity enhancing results of vaspin have been reported in humans, the full characterization of vaspin, and its functions have yet to be undertaken.

Resistin

Some of the adipokines secreted by adipocytes have deleterious effect on insulin sensitivity. One such adipokine is resistin, a 12 kDa peptide discovered during an examination of TZDs responsive genes in 2001 (60). Resistin has been reported to increase during adipogenesis as well as in insulin-resistant mice, and decrease upon TZDs treatment of mice. Moreover, antiresistin antibody treatment has been shown to improve insulin sensitivity and glucose transport *in vitro* and *in vivo* (61).

In humans, resistin has been shown to be expressed by macrophages. Studies investigating the association between resistin levels and insulin resistance in humans have shown variable results. However, resistin levels decrease after therapeutic treatment of diabetic patients with TZDs and metformin suggesting a role of resistin in insulin resistance in humans also (61).

Retinol Binding Protein 4

Retinol Binding Protein 4 or RBP4 is another adipokine with deleterious effects on insulin signaling and has been identified as a highly expressed circulating adipokine in insulin-resistant, adipose specific GLUT4 knockout mice and humans with obesity, and type 2 diabetes (62). It is expressed mainly in visceral adipose tissue supporting the hypothesis that visceral adipose tissue is associated with insulin resistance (63). In addition, correlation between serum RBP4 with RBP4 mRNA expression, visceral fat mass, total body fat mass, and insulin resistance has been reported (64). *In vivo* studies have shown that RBP4 overexpression or recombinant RBP4 induces insulin resistance whereas RBP4 deletion improves insulin sensitivity (5). However, other studies have failed to show any correlation of RBP4 with insulin resistance or obesity (61). Although these inconsistencies may be due to differences in patient populations or methodology, further studies are required to comprehend the features of RBP4.

Visfatin/PBEF/NAMPT

Visfatin is an adipokine that has been originally identified as a cytokine that promotes B cell maturation. Visfatin has been proposed to have a role in obesity and insulin resistance on the basis of studies in animal models. In mice, injection of visfatin decreases blood glucose levels whereas visfantin mutation leads to higher glucose levels. However, the role of visfatin in insulin resistance in humans is not clear due to conflicting results among different studies examining the plasma visfatin levels and obesity, insulin resistance and type 2 diabetes (5, 61). Therefore, further exploration is required to assess the role of visfatin in insulin sensitivity.

Inflammatory Cytokines

Adipose tissue is also a source of inflammatory cytokines such as TNF-α, IL-6, and Macrophage Chemoattractant Protein 1 (MCP-1). The role of TNF-α in the development of insulin resistance has been extensively studied, and is discussed in detail later in this thesis. Although, the association of IL-6 with obesity is not clear, some of the studies have shown the association of increased levels of IL-6 with impaired glucose disposal and insulin resistance. It has been speculated that IL-6 induces insulin resistance through cytokine signaling (SOCS) 1 and 3 that promotes Insulin Receptor Substrate (IRS) degradation (65). In addition to these inflammatory cytokines, adipose tissue

also secretes MCP-1, a chemokine that act as a chemoattractant to recruit macrophages to the adipose tissue. However, data on necessity of MCP-1 for macrophage infiltration in adipose tissue is conflicting. Kanda and colleagues have shown increased macrophage infiltration in adipose tissue and insulin resistance in MCP-1 expressing transgenic mice whereas MCP-1 deficient mice exhibited decreased macrophage infiltration in adipose tissue and improved insulin sensitivity when fed on the high fat diet (66). On the other hand, Inouye and colleagues have noted no difference in the number of macrophages recruited to the adipose tissue in MCP-1 deficient mice fed on high fat diet compared to the control mice (67).

Lastly, adipose tissue has also been shown to secrete Plasminogen Activator Inhibitor 1 (PAI-1). PAI-1 is expressed in the stromal vascular fraction of adipose tissue, and endothelial cells, and has been reported to increase in obesity. Prior evidences have supported the pro-thrombotic effects of PAI-1 due to its inhibitory action on plasminogen activators. PAI-1 has recently been linked to TSP1 expression in adipocytes. TSP1 is also thought to function as an adipokine, and has been observed to increase in obese, insulin resistant subjects with increased adipose tissue macrophage infiltration (61).

Thus, adipose tissue function is considerably more vast and complex than previously thought and actually functions as a full-fledged endocrine organ.

Moreover, the hormones secreted by adipose tissue interplay with one another, as well as with other tissues, and respond to nutrition to maintain glucose homeostasis. For example, adiponectin and TNF-α regulate the synthesis and activity of one another. Excess calorie intake and obesity have been theorized to activate inflammatory pathways that suppress adiponectin and increase inflammatory cytokines that ultimately lead to the disturbance of insulin signaling.

Insulin Signaling

Although adipokines play an important role in glucose metabolism, the most important hormone for glucose homeostasis is insulin. The isolation of insulin in 1921 was the greatest discovery that formed a foundation for future studies on glucose metabolism (68), in addition to providing a therapy for patients suffering from diabetes. Insulin is a small peptide hormone containing only 51 amino acids, and is secreted from the pancreas. The ability of insulin to lower blood glucose has been known for a long time, and the mechanism of insulin action on metabolism has been investigated intensely, yet complete understanding of the insulin signaling pathways and functions, has remained elusive.

The actions of insulin on insulin-target tissues mainly adipocytes, muscle, and liver, are mediated through the insulin receptor (IR). The IR is a

transmembrane tyrosine kinase receptor that upon insulin stimulation undergoes a conformational change, and autophosphorylation at multiple tyrosines (69-71). The phosphorylated tyrosines in the IR recruits scaffolding proteins, like IRS 1-4, that further recruit the downstream effector molecules (72-76). IRS1 and IRS2, bind the downstream signaling molecule – type 1A phosphatidylinositol 3-kinase (PI3K) (77) via its regulatory subunit (p85), and the binding of p85 to phosphorylated IRS activates the catalytic subunit of PI3K. The activated PI3K phosphorylates many phosphoinositides (PI), however type 1A PI3K preferentially phosphorylates PI (4,5) P_2 to form PI (3,4,5)P_3 that further recruits another serine/threonine kinase – 3-phosphoinositide-dependent protein kinase-1 (PDK1) (78-82). PDK1 together with PI3K is responsible for phosphorylation of another serine/threonine kinase – AKT/ Protein Kinase B (PKB) (83). AKT/PKB is also phosphorylated at Ser473, a site different from PDK1 phosphorylated Thr308, by a newly discovered protein kinase – mammalian target of rapamycin (mTOR) together with its regulatory subunit – RICTOR (84, 85). The signaling cascade downstream of AKT/PKB has been extensively studied, and plays vital role in the regulation of glucose transport, glucose and glycogen metabolism, lipolysis and lipogenesis, protein synthesis, and specific gene expression (86).

Regulation of glucose transport

The activation of AKT/PKB after insulin stimulation plays the key role in enhancing glucose uptake by increasing translocation of the glucose transporter GLUT4 from the cytosol to plasma membrane in insulin target tissues such as adipocytes and muscle. Among many glucose transporters, GLUT4 is the insulin responsive glucose transporter present in adipocytes and muscle, and the deficiency of GLUT4 specifically in the adipose tissue or muscle leads to insulin resistance in mice (87-89). GLUT4 mostly resides in a perinuclear storage site in the basal state (72, 90). However, insulin stimulation causes rapid exocytosis of GLUT4 vesicles from the perinuclear site to the plasma membrane. In addition, insulin also inhibits endocytosis of GLUT4 from the plasma membrane into the cytosol. Thus, an insulin-stimulated increase in exocytosis and a decrease in endocytosis of GLUT4 causes a dramatic increase of GLUT4 at the plasma membrane (72, 91-94). The increase of GLUT4 at the plasma membrane after insulin stimulation is also due to the inhibition of AS160. AS160 is a RAB-GAP protein that promotes intracellular retention of GLUT4 in the basal state. Upon insulin stimulation, AKT/PKB phosphorylates and inactivates AS160, thus allowing GLUT4 translocation to the plasma membrane (95, 96). The process of GLUT4 translocation to the plasma membrane is dependent upon cytoskeletal and motor structures such as microtubules, actin network, kinesins, and myo1C (90, 97-101). In addition to the trafficking of GLUT4

towards the plasma membrane, insulin also regulates the docking and fusion of GLUT4 with the plasma membrane via SNARE accessory proteins, Synip and Munc18 (92, 102, 103). Thus, insulin stimulated PI3K signaling cascade regulates GLUT4 translocation, docking and fusion to the plasma membrane, and the regulation of these processes by insulin occurs at multiple steps (93). Although, there are some reports of a PI3K independent pathway involved in glucose transport, the PI3K independent pathway is still controversial (99, 104).

Regulation of glycogenesis

Evidences show that the insulin mediated PI3K signaling cascade induces glycogenesis in muscle and liver. Glycogenesis is the process of glycogen synthesis mediated by the enzyme glycogen synthase (GS), that is responsible for the elongation of a glycogen chain by incorporating uridine 5'diphosphoglucose derived from glucose-6-phosphate (105). GS is phosphorylated and inhibited by the kinases such as PKA or glycogen synthase kinase (GSK). Studies have shown that insulin stimulation phosphorylates and inhibits GSK, and activates protein phosphatase1 (PP1) via PI3K, and AKT2 (106-108). It is thought that insulin mediated inhibition of GSK, and activation of PP1 result in decreased GS phosphorylation, and thus activation of GS (86, 109), thereby promoting glycogenesis.

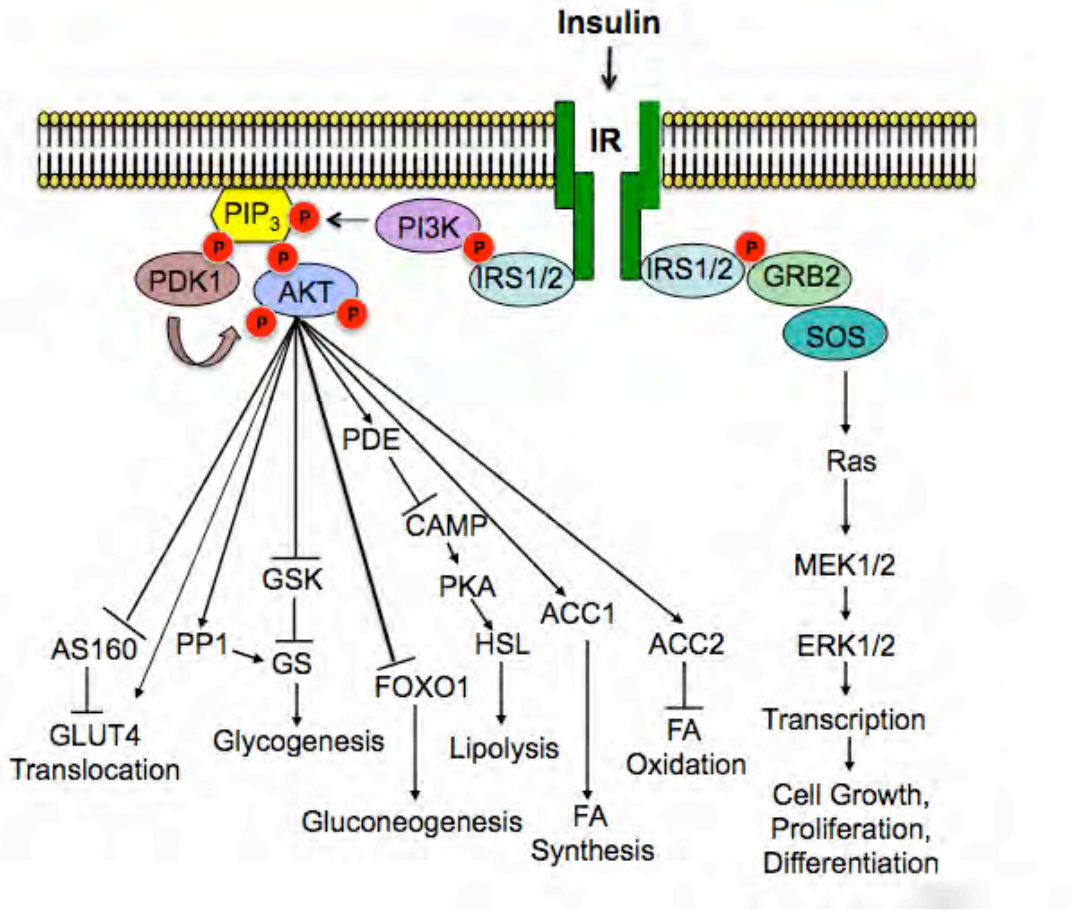

Fig.1.1 Insulin signaling pathways. The binding of insulin to the insulin receptor (IR) initiates a phosphorylation cascade that begins from the IR. Tyrosine phosphorylation of the IR recruits and phosphorylates insulin receptor substrate 1/2 (IRS1/2), which then bind and activate phosphatidylinositol 3-kinase (PI3K). PI3K phosphorylates PI (4,5) P_2 to form PI (3,4,5)P_3 that further phosphorylates downstream kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1). Phosphorylated PDK1 together with PI (3,4,5)P_3 activates AKT that mediates most of the insulin effects. AKT increases GLUT4 translocation directly as well as by inhibiting AS160, the inhibitor of GLUT4 translocation. AKT activation promotes glycogenesis via inhibition of GSK and activation of PP1. AKT also suppresses gluconeogenic gene expression by the inhibition of transcription factor FOXO1. Similarly, insulin signaling via AKT activates phosphodiesterase enzyme (PDE) to decrease cAMP levels, thereby inhibiting lipolysis. AKT promotes FA synthesis by activating Acetyl Co-enzyme A Carboxylase 1 (ACC1), and inhibits FA oxidation by activating Acetyl Co-enzyme A Carboxylase 2 (ACC2). The activation of IR by insulin also activates IRS1/2 to recruit GRB2 and SOS, thereby activating Ras/MEK1/2/ERK1/2 to promote cell growth, proliferation, and differentiation.

Regulation of gluconeogenesis

An important contributor to glucose homeostasis is the insulin-mediated regulation of gluconeogensis. Gluconeogenesis is the process by which liver synthesizes glucose from lactate, glycerol, and amino acids to maintain the serum glucose levels during prolonged fasting. It is, however, critical to inhibit this process of *de novo* glucose synthesis in the fed state in order to avoid glucose overload in serum, and this important function of inhibiting gluconeogensis after feeding is mediated by insulin. Prior studies have suggested that insulin signals via PI3K/AKT2 pathway to phosphorylate and inhibits FOXO1, and PPARγ co-activator 1α (PGC1α), which are the transcription factors responsible for the expression of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis, and glucose-6-phosphatase (G6Pase) (110-113). In addition to FOXO1 and PGC1α, insulin has also been shown to inhibit CREB binding protein (CBP), a co-activator of cAMP response element binding (CREB), and a transcription factor inducing gluconeogenic gene expression (114). These studies show that insulin negatively regulates the process of gluconeogenesis in the liver by suppressing the expression of gluconeogenic genes.

Regulation of Lipid Metabolism

The regulation of lipid metabolism by insulin is mainly through the effect of insulin in adipocytes to promote lipid storage and inhibit lipolysis. Prior

studies suggest that insulin increases storage of lipid in adipocytes in the form of triglycerides by promoting fatty acid reesterification, and triglyceride synthesis, as well as inhibiting fatty acid transport into mitochondria for α-oxidation (86). The fatty acid synthesis has been shown to be promoted by insulin-mediated activation of acetyl-coenzyme A carboxylase 1 (ACC1) whereas fatty acid oxidation is inhibited by insulin-mediated activation of acetyl-coenzyme A carboxylase 2 (ACC2) (115), thus giving the net result of fatty acid storage in adipocytes. In addition, insulin has also been shown to induce the activity of sterol regulatory element binding proteins-1c (SREBP-1c), a transcription factor involved in the expression of numerous enzymes involved in fatty acid and triglyceride synthesis, such as fatty acid synthase (FAS), steroyl-coenzyme A desaturase 1 (SCD1), acetyl-coenzyme A carboxylase (ACC), and glycerol-3-phosphate-1-acyltransferase (GPAT) (116). Insulin not only promotes fat storage, but it also inhibits lipolysis in adipocytes. Lipolysis in adipocytes is mediated through the activation of PKA by increased intracellular cAMP (discussed in detail in later sections). Previous studies have shown that insulin decreases intracellular cAMP by activating phosphodiesterase (PDE), an enzyme that converts cAMP to AMP, and thus inhibits lipolysis (117, 118).

Regulation of cell growth and differentiation

A different function of insulin compared to glucose and lipid homeostasis is the regulation of cell growth and differentiation (6, 119). It involves increases in protein synthesis, and the activation of transcription factors required for cell proliferation and differentiation (120-126). Evidences show that, insulin activates the transcription factors involved in cell proliferation and differentiation by stimulating the IR to recruit insulin receptor substrates, IRS and Grb2, which in turn bind to adaptor proteins, SHP2 and SOS, and together activate the serine kinase Ras (72, 86, 126, 127). Activation of Ras starts the phosphorylation cascade at MEK that finally activates ERK where it translocates into the nucleus to further activate transcription factors such as Elk1, sap1a, and Fra1.

In addition to the increase in transcription, insulin has also been shown to increase protein synthesis and decrease protein degradation (121-123). The increase in protein synthesis is thought to be mediated via PI3K and mTOR activation. This activation results in further activation of ATF4 which is responsible for regulating genes involved in amino acid biosynthesis and transporters (128). It is hypothesized that the translation of proteins is increased due to mTOR stimulated ribosomal biosynthesis, and inhibition of the negative regulator of protein synthesis 4E-binding protein-1 (4EBP1)

(128-132). Thus, the final outcome of increased protein synthesis after insulin stimulation allows cells to proliferate and differentiate.

Insulin, therefore, regulates a constellation of cellular functions mainly through the PI3K/AKT pathway or the Ras/MEK pathway, and there is cross-talk between these two pathways at multiple levels. Given the several important regulatory functions of insulin, it is predictable that any altercation in insulin signaling pathways resulting in insulin resistance will have a profound effect on whole body homeostasis. Thus, it is of the utmost importance to understand the biology of not only insulin signaling, but also insulin resistance.

Insulin Resistance

Insulin resistance involves a disturbance of the insulin signaling cascade and its resultant inability to lower blood glucose to a normal level of 4-7 mM. The persistent increase in blood glucose level above 7mM is the result of derangements in multiple physiological processes regulated by insulin such as glucose uptake in muscle and fat cells, suppression of gluconeogenesis in the liver, and promotion of glycogenesis in the liver and muscle. Though a thorough knowledge of all the perturbations that lead to insulin resistance has yet to be achieved, the vast amount of studies in past years have identified

several molecular mechanisms and mediators that contribute to the development of insulin resistance.

Molecular mechanisms of insulin resistance

The molecular mechanisms of insulin resistance includes defects at multiple levels of the insulin signaling cascade including decreases in the IR concentration, or defects in the IR kinase activity, decreases in IRS and PI3K concentration and phosphorylation, decline in GLUT4 concentration and translocation, and faulty intracellular enzymes and transcription factors (72, 86).

The IR is critical for the initiation of signaling cascade, and any defect in its activity may be due to genetic mutations or acquired changes in its phosphorylation state. The IR mutation, albeit rare, has been reported to cause insulin resistance of variable severity depending upon the ability of IR to hybridize with IGF-1 or other receptors (86).

Despite the presence of normal IR, modifications of the phosphorylation state of the IR to serine phosphorylation instead of tyrosine phosphorylation can down regulate insulin signaling. The serine phosphorylation of IR has been shown to decrease insulin signaling by inhibiting tyrosine phosphorylation, and promoting the association with 14-3-3 proteins (133, 134). Similarly,

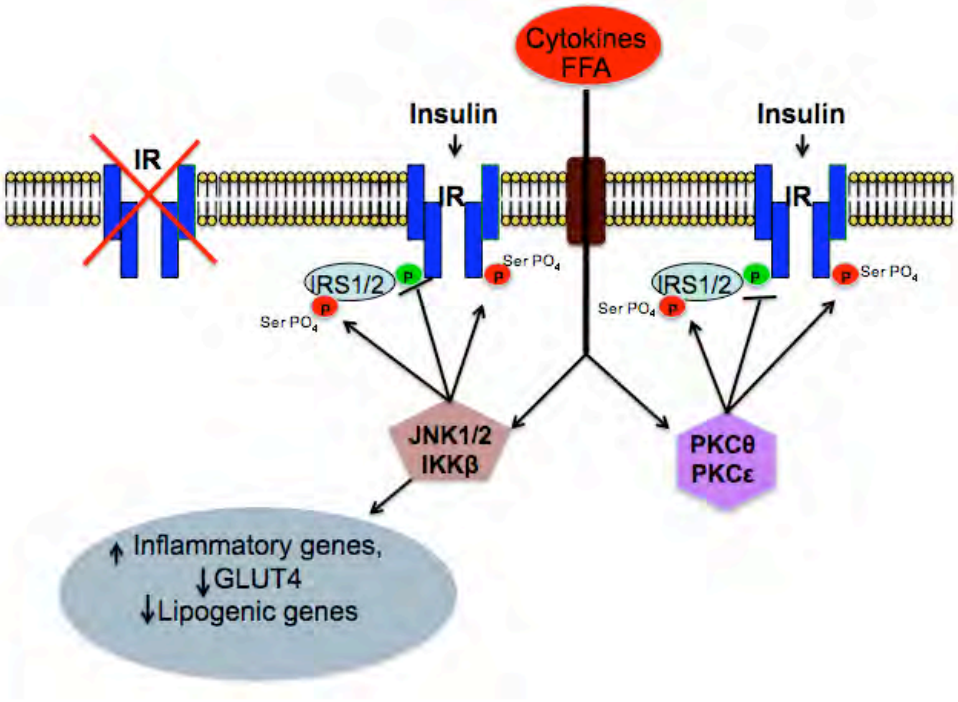

Fig.1.2 Mechanisms of Insulin Resistance. Insulin resistance may result from the decreased expression of Insulin receptor (IR) or mutations in IR. Insulin resistance may also result from the disruption of phosphorylation cascade from serine phosphorylation instead of tyrosine phosphorylation of IR and insulin receptor substrate (IRS1/2). The serine phosphorylation of IR and IRS1/2 are mediated by a number of kinases such as c-jun N-terminal kinase (JNK1/2), Ikappa B kinase (IKKβ), protein kinase Cθ (PKCθ), and protein kinase Cε (PKCε). The kinases JNK1/2 and IKKβ also decrease the expression of glucose transporter 4 (GLUT4), lipogenic genes, and increase the expression of inflammatory genes, thereby contributing to insulin resistance.

serine phosphorylation in place of tyrosine phosphorylation of IRS can also lead to diminished insulin signaling. This process involves a number of kinases such as c-jun N-terminal kinase (JNK), PI3K, GSK-3, AKT, mTOR, protein kinase C (PKC), and IKappa kinase B (IKKB) (72, 135, 136). Alterations of these kinases *in vivo* have been shown to modify insulin sensitivity. For example, in rodents, overexpression of IKKB in the liver leads to insulin resistance in liver and muscle whereas IKKB deletion confers protection against diet-induced hepatic insulin resistance (137). In contrast to the kinases, IRS function can also be inhibited by the action of phosphatases that dephosphorylate IRS or IR as well. One of the phosphatases protein tyrosine phosphatase 1 B (PTB1B), has been speculated to inhibit insulin signaling as demonstrated by mice deficient in PTB1B. Mice deficient of PTB1B are more insulin sensitive, and are resistant to diet-induced obesity (138). Besides kinases and phosphatases that modulate IRS phosphorylation, IRS signaling can also be inhibited by degradation of IRS induced by binding of suppressor of cytokine signaling 1 and 3 (SOCS1 and SOCS3) to IRS (139).

The irregularities in the effectors downstream of the IR and IRS can also fuel insulin resistance. For example, absence of AKT2 in mice has been shown to cause insulin resistance in the liver and muscle (140). Similarly, adipose

tissue and muscle specific GLUT4 knockout mice also have impaired glucose tolerance, and insulin resistance (141, 142).

Mediators of Insulin Resistance

Apart from probing the molecular aspects of insulin resistance, a lot of effort has been made on exploring the mediators of insulin resistance. Unraveling these mediators would assist to understand how molecular derangements of insulin signaling develop from the harbingers of insulin resistance, especially over-nutrition and obesity.

Obesity is the increase in body weight particularly due to the accumulation of body fat that results from an imbalance between calorie intake and energy expenditure. Although obesity awareness is increasing currently, obesity has been recognized to be more than a progression of physiological storage of energy, and has been categorized as a chronic disease since National Institutes of Health Consensus Conference in 1985 (143). Furthermore, in 1986, the Framingham Heart Study has shown the risk of death within 26 yr increases by 1% for each extra pound added to the weight of an individual between 30 to 42 yr of age, and the risk increases to 2% for each pound gained between 50 to 62 years of age (144). In fact, future life expectancy, the predictor of health and health care, may decline for the first time due to the escalation of obesity incidence (145). Although obesity is associated with

numerous chronic diseases such as osteoarthritis and fatty liver disease, the most common diseases correlating with obesity are cardiovascular diseases and insulin resistance, which constitute the metabolic syndrome. Studies trying to elucidate the progression of obesity to insulin resistance, and metabolic syndrome have identified mediators such as adipokines, inflammatory cytokines, lipids, and FFAs that emphasizes the importance of adipose tissue in maintaining the whole body glucose metabolism.

Adipokines are the hormones secreted by the adipose tissue (discussed in detail in “Adipose tissue as an endocrine organ” section). In obesity, the balance between adipokines conferring insulin sensitivity and insulin resistance is disturbed. It is observed that secretion of adipokines such as adiponectin that promote insulin sensitivity, are decreased in obese individuals whereas adipokines contributing to insulin resistance such as resistin, RBP4, and inflammatory cytokines such as TNF-α and IL-6 are increased, thus creating a pro-insulin-resistant environment.

Inflammatory cytokines

Prior evidences have shown increase in the circulating levels of inflammatory cytokines, and macrophage infiltration in the adipose tissue in obesity, suggesting the association of obesity with chronic low-grade inflammation (146). Although the etiology of inflammation in obesity is not known, the role

of inflammatory cells, particularly macrophages, has emerged after the detection of resident macrophages in the adipose tissue (147, 148). More macrophages are present in adipose tissue in obese individuals probably due to increased expression of MCP-1 by hypertrophied adipocytes (149). These macrophages fuse to form a ring-like structure around dead adipocytes known as “crown-like structure’’ in order to clear dead cells (150). These “crown-like structures” are considerably increased in obesity suggesting increased adipocyte necrosis in obesity (151). The causes of adipocyte necrosis may be several, and hypoxia due to expansion of adipose tissue with reduced blood supply is one of the proposed causes (152, 153). Nevertheless, the activation of infiltrated macrophages in adipose tissue, and/or hypoxia leads to the increased expression of inflammatory cytokines such as TNF-α and IL6 (154-157).

TNF-α

Among the inflammatory cytokines that are elevated in obesity, TNF-α has been extensively studied, and its association with obesity induced insulin resistance has been well established. The levels of TNF-α in the serum has been reported to correlate with body weight (158), and is increased in obesity. Similarly, increased expression of TNF-α has been detected in adipose tissue from obese diabetic rodents, and has been hypothesized to mediate insulin resistance (159). Consistently, circulating TNF-α, and local gene expression

of TNF-α in adipose tissue are increased in obese humans also (160), and acute infusion of TNF-α in healthy humans has been shown to increase insulin resistance (161). Furthermore, inhibition of TNF-α function by genetic deletion of TNF-α or TNF-α receptor (TNFR) has been shown to prevent obesity induced insulin resistance (162-164). However, neutralization of TNF-α with TNF-α antibody improves insulin sensitivity only in rodents, but not in humans (159, 165). Despite the discrepancy of the effect of TNF-α antibody on insulin sensitivity in rodents and humans, evidences indicating the role of TNF-α in obesity related insulin resistance are substantial.

Although the adipocyte expression of TNF-α is increased in obesity, a more important source of TNF-α from the fat tissue is considered to come from the cells in the stromal vascular fraction, and infiltrated macrophages (147, 148, 166-170). The expressed TNF-α protein exists as a 26 kDa transmembrane monomer, and a smaller 17 kDa soluble TNF-α formed after proteolytic cleavage of membrane TNF-α. Both membrane bound and soluble form of TNF-α can mediate the metabolic effects of TNF-α via stimulation of the TNFR (166). Between the two TNFRs, expressed ubiquitously, TNF-α receptor-1 (TNFR1) mediates most of the TNF-α actions in adipocytes. It has been shown that deletion of TNFR1 improves insulin sensitivity *in vitro* and *in vivo* (171-173). The activation of TNFR initiates several cellular signals that may contribute to the development of insulin resistance.

The binding of TNF-α to TNFR1 activates several pathways to activate kinases such as JNK, extracellular signal-related kinase (ERK), PKC, and IKKB, which inhibit insulin-stimulated tyrosine phosphorylation of both the IR and IRS, thus impairing insulin function. TNF-α signaling in adipocytes also suppresses a number of genes required for insulin signaling such as IR, IRS and GLUT4. In addition to these genes, TNF-α also suppresses lipogenic genes and adiponectin probably via suppression of the master transcription factor PPARγ. Altogether, the effect of TNF-α on transcription in adipocytes results in decreased adipocyte differentiation and a decrease in TG accumulation. (See reference (135, 166) for more detailed review).

Lipolysis in Adipocytes

In the low-grade inflammatory state of obestiy, TNF-α is hypothesized to be the main mediator of lipolysis. Lipolysis is a process by which various lipases hydrolyze TG in lipid droplets to glycerol and FFAs. One of the proposed mechanisms by which TNF-α increases lipolysis in adipocytes is the suppression of perilipin that coats the lipid droplets to protect against lipases (174). The suppressive effect of TNF-α on perilipin may be via PPARγ, which drives the transcription of perilipin in adipocytes (16). TNF-α has also been shown to reduce the level of G_i protein with a function of inhibiting lipolysis (175). Another inhibitor of lipolysis is PDE, and TNF-α has been implicated in

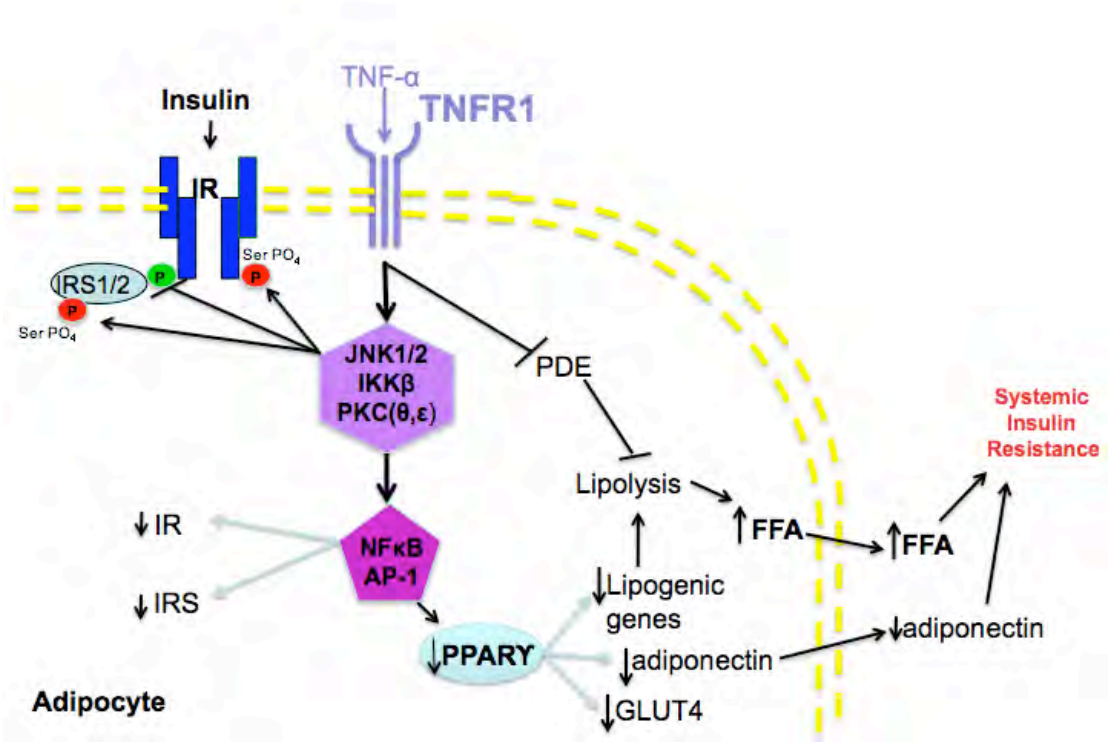

Fig. 1.3 TNF-α mediated insulin resistance. TNF-α acts via the TNF-α receptor (TNFR1) to activate downstream kinases such as c-jun N-terminal kinase (JNK1/2), IKappa B kinase (IKKβ), and protein kinase C (PKC θ,ε). These kinases phosphorylate insulin receptor (IR) and insulin receptor substrate (IRS1/2) at serine residues that disrupts the insulin signaling cascade. JNK1/2 and IKKβ also suppress the expression of IR and IRS via NFκB and AP-1 transcription factors. NFκB and AP-1 also suppress PPARγ, thereby decreasing the expression of lipogenic genes, adiponectin and glucose transporter (GLUT4). TNF-α also suppresses the expression and activation of phosphodiesterase (PDE). The inhibition of PDE together with suppressed lipogenic genes increases lipolysis, thus releasing free fatty acids (FFA) into the circulation. Elevated FFA and decreased adiponectin both contribute to the development of systemic insulin resistance.

the decrease in the expression and activation of PDE (176). In addition to these, TNF-α may also mediate lipolysis by disruption of the insulin signaling cascade, and inhibition of the anti-lipolytic actions of insulin. All of the above effects of TNF-α lead to the increases in lipolysis in adipocytes. Despite the discovery of various molecular mechanisms of TNF-α mediated lipolysis, the complete understanding of all of the mechanisms of multifunctional TNF-α has remained elusive.

The increase in adipocyte lipolysis in obesity can also be due to the increased adipocyte size. Previous studies have reported that the rate of lipolysis correlates with the size of adipocytes, and the level of pro-lipolytic signaling molecule, cAMP, is higher in larger adipocytes (177, 178). Moreover, it has also been shown that hypertrophied adipocytes have decreased insulin sensitivity, and thus have decreased insulin-mediated anti lipolytic effect (179).

Unlike the hypertrophy of adipocytes and the effects of TNF-α to mediate lipolysis in obesity, the physiologic stimuli for lipolysis are catecholamines: adrenaline and nor adrenaline. These hormones are released during fasting and bind to β adrenergic receptors that are coupled to G proteins. The activated G proteins further activate adenylate cylase, an enzyme that converts ATP to cAMP, thus increasing intracellular cAMP levels. The

elevated cAMP ultimately activates cAMP dependent protein kinase, PKA (135, 177, 180, 181). Activation of PKA then phosphorylates perilipin, and hormone-sensitive lipase (HSL), which can then interact and enhance lipolysis (180, 182-186). HSL is a lipase that preferentially hydrolyzes diacylglycerols (187), and is required for normal adipose tissue function. It has been observed that HSL depleted mice have reduced adipose tissue, and a decreased lipolytic response to catecholamines (188, 189). Another lipase, adipocyte triglyceride lipase (ATGL), has also been demonstrated to be required to mediate lipolysis in response to catecholamines (184, 190-192), and catalyzes the initial step in TG hydrolysis (192, 193). Similar to HSL deficient mice, ATGL deficient mice have also been shown to exhibit defective lipolysis after catecholamine treatment (190).

Irrespective of the stimuli for lipolysis, the TG hydrolysis leads to the release of glycerol and FFAs that efflux from adipocytes into the circulation. These released FFAs provide fuel to tissues such as muscle and liver during fasting when serum glucose levels are low. However, with chronic and excessive lipolysis, serum FFA levels are elevated causing lipotoxicity, and thereby insulin resistance. The mechanism of FFA-induced insulin resistance has been hypothesized to be caused by the inhibition of glucose uptake, glycogen synthesis and glucose oxidation as well as by increasing hepatic glucose output (86, 194). Increased FFA is also associated with diminished insulin

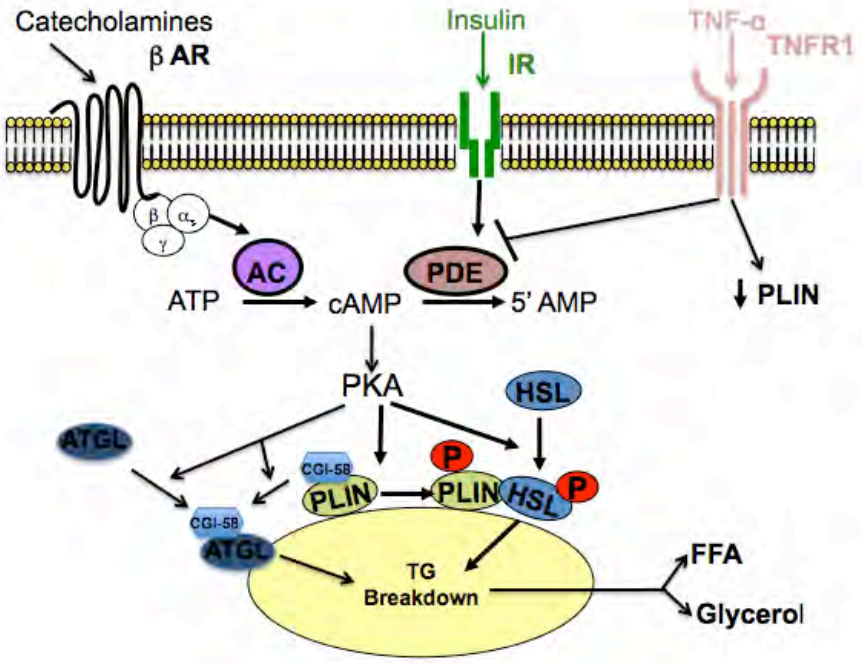

Fig. 1.4 Regulation of Lipolysis in adipocytes. Catecholamines activate adenylate cyclase (AC) via β adrenergic receptor (β AR). Activated AC converts ATP to cAMP that further activates cAMP activated protein kinase A (PKA). Once activated PKA phoshorylates perilipin (PLIN), which acts as a docking site for phosphorylated hormone sensitive lipase (HSL). The phosphorylation of perilipin releases CGI-58 that then interactes with ATGL (Adipocyte Triglyceride Lipase) and promotes ATGL lipase activity The action of HSL and ATGL on triglycerides (TG) releases glycerol and free fatty acids (FFA). TNF-α decreases the expression of PLIN that removes the protective covering of lipid droplets, and inhibits phophodiesterase (PDE) to increase cAMP, thus promoting lipolysis. Insulin activates insulin receptor (IR), and then activates PDE, which decreases cAMP levels, thereby inhibits lipolysis.

stimulated IRS-1 phosphorylation and PI3K activity (195). On the other hand, higher levels of circulating FFAs also promote accumulation of TG and fatty acid metabolites such as diacylglycerol (DAG), fatty acyl CoA and ceramide accumulation in the liver and muscle tissue. It has been observed that ectopic accumulation of TG in muscle correlates with insulin resistance in patients with obesity and diabetes (196). Despite the limited knowledge of how FFAs mediate insulin resistance, it is apparent that FFA metabolism and regulation have an impact on maintaining whole body insulin sensitivity.

Lipid droplets and associated proteins

To prevent the deleterious effects of elevated circulating FFAs, these are sequestered and stored in the form of TG in lipid droplets in adipocytes. The lipid droplets are now considered as a dynamic organelle rather than simply a lipid storage unit. The lipid droplet consists of a central core of neutral lipids, mainly TGs and some choleresterol esters, surrounded by a monolayer of phosholipids. Although, the formation of lipid droplets is still under investigation, the current theory suggests that TGs are synthesized in the endoplasmic reticulum (ER) through a process of enzymatic esterification. This theory is supported by studies that show numerous enzymes involved in TG biosynthesis such as acyl–CoA synthetase (ACS), glycerol-phoshate acyltransferase (GPAT), acylglycerol-phosphate acyltransferase (AGPAT),

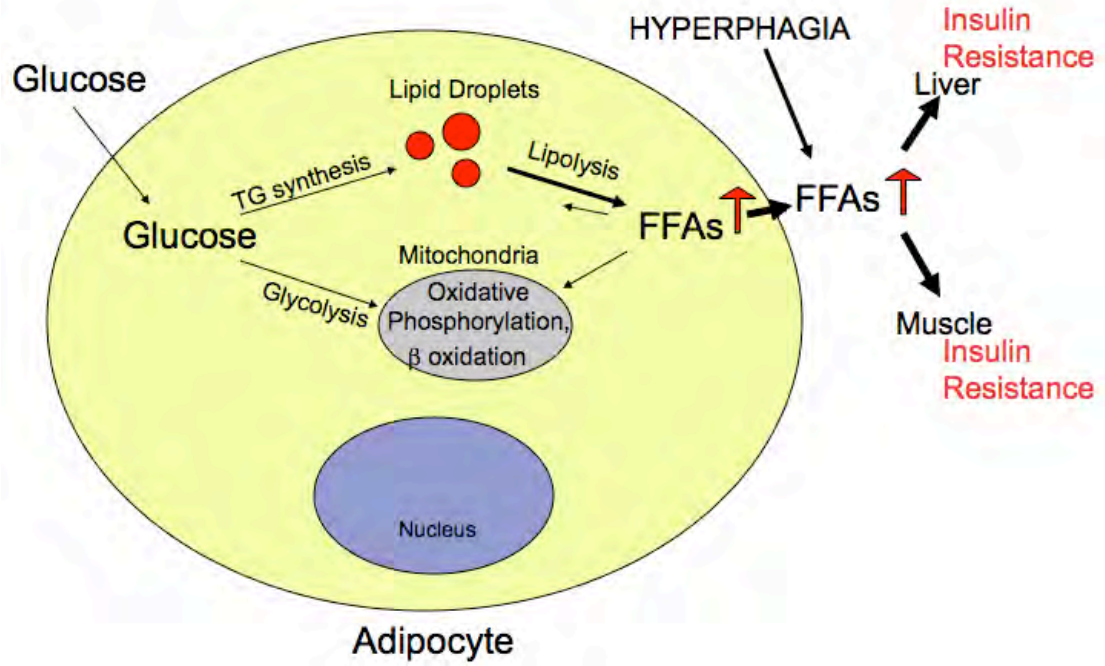

Fig 1.5 Role of the Adipocyte in the regulation of free fatty acids (FFAs). The circulating glucose is taken up by adipocytes where the glucose either undergoes glycolysis in the cytosol, and then oxidative phosphorylation in the mitochondria or undergoes triglyceride (TG) synthesis to be stored in lipid droplets. TG in lipid droplets can undergo lipolysis to release FFAs during fasting. Increased circulating FFAs during hyperphagia can enter adipocytes either to be stored in lipid droplets or to be β oxidized in mitochondria. In obesity, the balance between lipolysis and storage is disturbed so that more FFAs are released. These FFAs efflux into the circulation, and mediate insulin resistance in other tissues such as the liver and muscle tissue.

and diacylglycerol acyltransferase (DGAT) fractionate with the ER, and show that these enzymes reside in the ER (116, 197-201). In addition, TG droplets are surrounded by or even seen continuous with the ER suggesting the ER as a site of origin of lipid droplets (202-205). It is speculated that the newly synthesized triacylglycerols accumulate to form a disc in the interior of the ER bilayer. Increase in the size of the lipid disc leads to the budding off of the lipid disc along with the monolayer of the ER membrane. However, the phospholipid monolayer on the surface of the lipid droplet is distinct from the phospholipid layer of the ER suggesting that the budding of lipid droplets from the ER may occurs at the specialized subdomains of the ER, or may be a completely different process (206) The growth of lipid droplet size is also not fully understood. It is theorized that the emerging small lipid droplets bud off from the ER, and are directed towards existing cytosolic lipid droplets where the smaller droplets fuse with the existing larger droplets, or transfer TG to the larger droplets (197). It is also possible that the existing lipid droplets grow in size by synthesizing TGs themselves because the enzymes involved in TG synthesis are present in these lipid droplets as well.

The lipid droplets consist of a core of hydrophobic TGs surrounded by a phospholipid monolayer, and proteins. The proteins surrounding the lipid droplets are different depending upon the cell type, metabolic condition, and even different between lipid droplets within a cell (207). Previous studies have

identified several proteins associated with lipid droplets such as perilipin, adipocyte related protein (ADRP), tail-interacting protein of 47 kDa (TIP47), S3-12, OXPAT, Cell Death-inducing DFF45-like effector proteins (CIDE proteins), and various lipases. The lipases such as HSL and ATGL may associate with the lipid droplet surface in the lipolytically active state as described earlier in “Lipolysis in Adipocytes” section. The other lipid droplet proteins were grouped together as “PAT” family of proteins after the discovery of the first three members: perilipin, ADRP, and TIP47. They share common domains that are thought to be responsible for lipid targeting and anchoring. These proteins are further sorted into two groups: Exchangeable TG associated PAT proteins (EPATs) and Constitutively TG associated PAT proteins (CPATs). EPATs consist of TIP47, S3-12 and OXPAT, and are constitutively present in the cytosol, and are thought to coat lipid droplets as they emerge from the ER. On the other hand, CPATs are found only bound to the lipid droplets because the unbound free CPATs are rapidly degraded. Perilipin and ADRP are two proteins grouped as CPATs (197).

Perilipin

Perilipin is the most extensively studied lipid droplet associated protein. Of the three isoforms of perilipin: perilipin A, B, and C, perilipin A is the most abundant, and thus the most commonly studied lipid droplet protein. Not only is perilipin highly expressed in adipocytes, it is also considered a marker of

adipocyte differentiation. In addition, the expression of perilipin in adipocytes is under the control of PPARγ, the adipogenic transcription factor (16). Once expressed and targeted to the lipid droplet, perilipin is very stable with a half-life of 40h (208), however, the unbound perilipin is rapidly degraded. Two groups studying the mechanism of perilipin degradation showed different results: Xu *et. al.* indicated proteasomal degradation, while Kovsan *et. al.* suggested lysosomal degradation (209-211).

Perilipin surrounding the lipid droplets is postulated to form a protective coat, and decrease the rate of TG hydrolysis (212-214). Loss of perilipin under conditions such as TNF-α treatment promotes lipolysis (174). In addition, *in vivo* rodent models lacking perilipin also have increased basal lipolysis (215, 216). Interestingly, transgenic mice overexpressing perilipin in the adipose tissue exhibit protection against diet-induced obesity, despite the propensity of perilipin to store fat (217). However, the overexpression of perilipin in adipocytes protects lipid droplets from lipolysis stimulated by TNF-α, but not by catecholamines (174). In fact, previous studies have shown that the phosphorylation of perilipin by catecholamines is a necessary event for catecholamine-mediated lipolysis, and is further supported by the data from the perilipin null mice that have attenuated stimulated lipolysis (215, 216). The phosphorylation of perilipin has been avidly researched, and has been shown to be involved in multiple aspects of lipid metabolism. For example,

phosphorylation of perilipin at Ser517 is essential for the activation of ATGL activity, and lipolysis (218). On the other hand, perilipin promotes HSL-mediated hydrolysis via both phosphorylation dependent, and independent mechanisms (219, 220). Moreover, phosphorylation of perilipin at Ser492 is responsible for lipid droplet fragmentation, and dispersion after catecholamine stimulation. In addition, perilipin also controls lipid metabolism by interacting with other proteins such as CGI-58, an activator of ATGL (221, 222). The importance of perilipin in metabolism is exemplified by the association of increased risk of diabetes with several SNPs detected in the perilipin gene (207).

ADRP

ADRP also known as adipophilin, like perilipin, has also been found to be constitutively bound to lipid droplets. In the free form ADRP is rapidly ubiquitinated to be degraded via the proteasome (223). ADRP is expressed early during adipogenesis, but its expression declines during maturation and is ultimately replaced by perilipin in mature adipocytes (197). In addition to adipocytes, ADRP is also found in other cells that accumulate lipid, e.g. macrophages (224). Although the ADRP-null mice has normal adipogenesis and adipose tissue, the TG content is reduced in hepatocytes, and these mice are resistant to hepatic steatosis induced by high fat feeding (225). Conversly, the overexpression of ADRP promotes TG accumulation in

different types of cells like fibroblasts, COS and hepatocytes in culture (226-228). It has been shown that ADRP expression is increased upon isoproterenol treatment of adipocytes via a PKA independent mechanism (229). Although ADRP is post-translationally modified, the implication of such modification has not been uncovered.

TIP47, S3-12, OXPAT

TIP47 (PP17/M6PBP), S3-12 and OXPAT (MLDP, LSDP5) are stable lipid droplet proteins that can shuttle between lipid droplets and the cystosol. TIP 47 is ubiquitously expressed, whereas S3-12 is preferentially present in adipocytes, and OXPAT is primarily expressed in the heart, skeletal muscle and liver. Although these proteins are not studied in detail yet, it has been shown that these proteins can be associated with lipid droplets, and promote TG accumulation (197, 207).

CIDE Proteins

The newest proteins to emerge in the field of lipid droplet proteins are CIDE proteins. CIDE proteins have three isoforms: CIDEA, CIDEB, and CIDEC (FSP27). These proteins have domains homologous to DNA fragmentation factor 45 (DFF45) (230), but more importantly have domains homologous to perilipin, and are thought to be responsible for lipid droplet targeting, anchoring, and shielding (231). Recently, our lab has shown both CIDEA and

CIDEC are targeted to lipid droplets (232, 233), and subsequently has initiated intensive interest in these proteins. Although some studies have shown the effect of CIDE proteins in apoptosis by virtue of its DFF45 domain, high expression of CIDEC in adipocytes does not cause cell death (234, 235), and increasing evidence supports their more important role in the lipid metabolism.

CIDEA is highly expressed in the mouse BAT, and its depletion in mice leads to protection against diet-induced obesity and insulin resistance. Two different studies by the same group published that CIDEA binds and represses UCP1 in the mitochondria (236), and then later published that CIDEA is localized to the ER, and suppresses AMP-activated protein kinase (AMPK) activity (237). However, later studies have confirmed the localization of CIDEA in lipid droplets instead of mitochondria (231). Similar to other lipid droplet proteins, the expression of CIDEA is controlled by PPARγ and PPARα (17). CIDEA is rapidly ubiquitinated, and degraded via the proteasome (238). In contrast to rodents, CIDEA is highly expressed in the human WAT. It has been shown that CIDEA expression is down regulated in obese humans, and increases after weight loss. CIDEA expression is also observed to decrease with TNFα treatment, and the decrease in CIDEA is associated with increased lipolysis (239, 240). In addition, the expression of CIDEA is decreased in obese insulin resistant individuals compared to obese insulin

sensitive ones (231). Taken together, these data suggest a role for CIDEA in lipid metabolism, and is further supported by the detection of CIDEA V115F polymorphism association with obesity in Swedish and Japanese population (241, 242).

CIDEB is mainly expressed in the hepatocytes. Studies with CIDEB null mice have shown decreased lipogenesis in hepatocytes, and resistance to obesity in response to a high fat diet. These mice also have increased insulin sensitivity, and hepatic fatty acid oxidation (243). CIDEB is reported to interact with apolipoprotein B to promote the formation of TG-enriched very low-density lipoproteins (VLDL) (244). In addition, CIDEB has also been shown to be involved in cholesterol metabolism in the liver (245).

CIDEC, also known as Fat Specific Protien 27 (FSP27) in mice, is preferentially expressed in white adipocytes. The expression of FSP27 is under the transcriptional control of PPARγ2 (246). The expression of FSP27 increases after treatment with the PPARγ agonist, rosiglitazone, (a common medication used with type 2 diabetic patients) (247). FSP27 is targeted to lipid droplets via its CIDE C domain (248), and promotes lipid accumulation, and unilocular lipid droplet formation *in vitro* and *in vivo*. Consistently, depletion of FSP27 in adipocytes in culture, or in mouse models leads to lipid droplet fragmentation, and lipolysis (233, 234). It is proposed that FSP27

enhances lipid accumulation by decreasing β oxidation of fatty acids (249). It is also observed that FSP27-null mice have increased mitochondrial activity, and the white adipocytes become more like brown adipocytes (234, 250). In addition, FSP27 depleted mice are resistant to diet-induced obesity, and insulin resistance. The increased insulin sensitivity in FSP27 deficient mice has been reported to be associated with an increase in IRS-1 and AKT phosphorylation, and an increase in GLUT4 expression in adipose tissue (250). However, a CIDEC homozygous nonsense mutation in human that is analogous to FSP27 knock-out mice is associated with lipodystrophy and insulin resistance where as FSP27 knock-out mice are insulin sensitive (251). It can be argued that FSP27 null mice are lacking FSP27 completely, whereas the mutated CIDEC in humans are still expressed but are unable to target to lipid droplets. Perhaps the presence of mutated FSP27 does not allow the increase in β-oxidation, and IRS-1/AKT phosphorylation that may contribute to improved insulin sensitivity in mouse models. Though, human and mouse models are different in the FSP27 knock-out aspect, both human and mouse models have decreased FSP27 expression associated with obesity. Both *ob/ob* and high-fat diet induced obese mice have decreased expression of FSP27 as the obesity progesses and decreased insulin sensitivity ensues. In case of obese humans, CIDEC protein is observed to be down regulated in the insulin resistant obese patients compared to the

insulin sensitive obese patients (231). Taken together, these data suggest that FSP27/CIDEC has an important role in lipid metabolism.

Specific Aims:

Previous studies have shown the association of elevated circulating FFAs with obesity and insulin resistance (252). The increase in circulating FFAs has been speculated to contribute to insulin resistance in humans and mice whereas decrease in circulating FFAs by an antilipolytic drug acipimox improves insulin sensitivity (253-255). Studies have demonstrated that the higher circulating FFA levels also promote ectopic accumulation of TG in the muscle, that may contribute to insulin resistance (135). These FFAs in circulation may come from ingested food as well as from the adipose tissue via lipolysis. Lipolysis can be stimulated by catecholamines during fasting, or by cytokines such as TNF-α in the state of chronic inflammation and obesity (180, 256). On the other hand, these FFAs can be sequestered and stored as TG in lipid droplets in adipocytes. Thus, the functional integrity of adipose tissue is essential, perhaps to prevent the adverse effects of FFAs. The deficiency of adipose tissue in both rodents and humans has been shown to be related to the higher circulating levels of FFAs, and insulin resistance (20-23).

In adipocytes, the balance between lipogenesis in the fed state, and lipolysis in the fasting state is finely controlled by several regulators including proteins associated with lipid droplets. One of the lipid droplet proteins shown to promote lipid accumulation *in vitro* and *in vivo* is FSP27. FSP27 is a novel lipid-droplet associated protein. FSP27 is observed to be decreased in the obese insulin resistant individuals compared to the obese insulin sensitive ones (231). In addition to this, FSP27 mutation has been reported in humans with partial lipodystrophy and insulin resistance (251). In contrast to human data, it is the absence of FSP27 that promotes insulin sensitivity in rodents (234). On the other hand, FSP27 depletion leads to formation of smaller lipid droplets, and lipolysis in both humans and rodents (233, 235, 249, 257). Although previous studies have suggested the role of FSP27 in lipid metabolism, and insulin sensitivity, not much is known about the regulation of FSP27, and the effect of FSP27 alteration in lipid metabolism. Understanding the regulation of FSP27 in normal and obese conditions, and its role in lipid metabolism will provide new insights to the present knowledge of lipid metabolism. Therefore, this study explores the role and regulation of FSP27 in the basal state as well as during lipolysis in adipocytes.

The specific aims of this study are:

1. To determine how FSP27 is regulated in the basal condition. This study and a recently published study show that FSP27 has a half life of

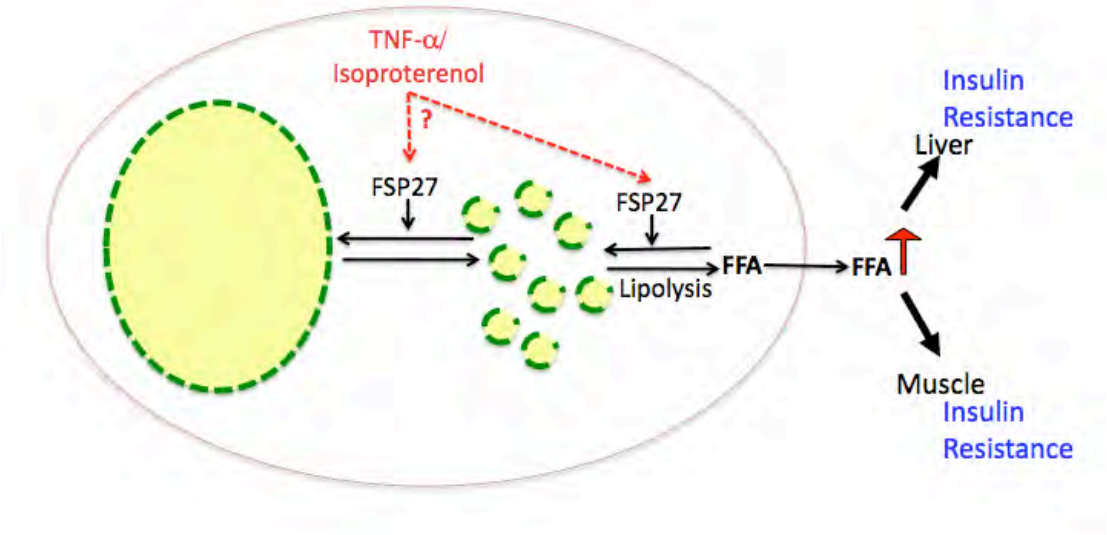

Fig. 1.6: Investigating the effect of TNF-α and isoproterenol on FSP27 and its effect on lipolysis. FSP27 has been shown to promote unilocular lipid droplet formation by decreasing lipolysis. The work presented in this thesis investigates if there is an effect of lipolytic stimuli such as TNF-α and isoproterenol on FSP27, and if there is an effect of such stimuli on FSP27 what is the implication of FSP27 regulation by these stimuli on overall lipolysis.

less than 15 min because it is rapidly ubiquitinated, and degraded via the proteasome (258).

2. To test if TNF-α, the cytokine elevated in the obese state, has any effect on FSP27, and if the effect of TNF-α on FSP27 plays a role in the lipolysis mediated by TNF-α. This study identifies the depletion of FSP27 by TNF-α as a novel mechanism of TNF-α mediated lipolysis.
3. To examine if the physiological stimuli of lipolysis, catecholamine, regulates FSP27, and the role of catecholamine mediated regulation of FSP27 in stimulated lipolysis. This study shows catecholamines upregulates FSP27 by decreasing FSP27 ubiquitination, and degradation. These results are also consistent with a recently published study (258). This study further shows the paradoxical increase of FSP27 after catecholamine stimulation may be due to a feedback mechanism to protect adipocytes from the potent lipolytic effect of catecholamines.

Chapter II

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CONTRIBUTIONS

The author performed all the experiments in this study. Pallavi Gandhi and Anil Chawla made the adenoviral HA tagged FSP27 construct. Emilie Boutet performed the experiment in Fig. 2.13 C and D.

Some part of this chapter (Fig. 2.1) is published in: Puri V, **Ranjit S**, Konda S, Nicoloro SM, Straubhaar J, Chawla A, Chouinard M, Lin C, Burkart A, Corvera S, Perugini RA, Czech MP. Cidea is associated with lipid droplets and insulin sensitivity in humans. Proc Natl Acad Sci U S A. 2008; Jun 3; 105(22):7833

Summary

FSP27, a lipid droplet-associated protein, functions to suppress lipolysis and thereby enhance triglyceride accumulation in adipocytes (233, 234, 249). Our result is consistent with the literature showing FSP27 is under the control of the transcription factor PPARγ, the master regulator of adipogenesis (246). FSP27 was recently found by us and others to be a remarkably short-lived protein, with a half-life of only 15 min, due to its rapid ubiquitination and degradation by the proteasome (258).

As FSP27 has a rapid turnover in adipocytes, we tested the hypothesis that the regulation of FSP27 protein levels is a mode by which the lipolytic agents TNF-α, a cytokine, and isoproterenol, a β-adrenergic agonist, act to regulate FFA release from adipocytes. Consistent with this concept, we report here that TNF-α markedly and rapidly decreases FSP27 mRNA and protein levels, and in parallel decreases lipid droplet size and increases glycerol release. Similar depletion of FSP27 using siRNA mimicked the action of TNF-α to enhance lipolysis, while prevention of the decrease in FSP27 protein by TNF-α using adenovirus-directed expression of FSP27 blocks the effects of TNF-α on lipolysis and lipid droplets size. Analysis of the time course during TNF-α treatment of cultured adipocytes revealed that the decrease in FSP27 protein levels precedes the decrease in lipid droplet size, which in turn is followed by

increased lipolysis. As expected, the lipase, ATGL, is required for the increased lipolysis caused by either FSP27 depletion or TNF-α treatment. Together these data identify FSP27 as an important intermediate in the mechanism of TNF-α actions on two distinct processes of diminishing lipid droplet size, and stimulating lipolysis in cultured adipocytes.

In contrast to the effect of TNF-α on FSP27, the much greater lipolytic action of isoproterenol is paradoxically associated with increases in cellular FSP27 protein levels, and a delayed degradation rate corresponding to decreased ubiquitination. These results are consistent with a recently published study (258). The catecholamine-mediated increase in FSP27 abundance is mimicked by Forskolin or soluble 8-Bromo-cAMP treatment, and prevented by PKA inhibitor KT5720 or PKA depletion using siRNA. Despite the necessity of PKA for isoproterenol-mediated stabilization of FSP27, PKA does not phosphorylate FSP27 suggesting an involvement of another protein or proteins in the stabilization of FSP27 in response to isoproterenol. The results in this study also show that isoproterenol increases cellular FSP27 levels despite blunted lipolysis in adipocytes transfected with ATGL siRNA, suggesting that lipolytic effect of catecholamines is not required for catecholamine mediated stabilization of FSP27. Furthermore, adenovirus directed expression of FSP27 is able to protect against isoproterenol mediated lipid droplet size diminution. This data indicates that depletion of

FSP27 plays a role in catecholamine-mediated lipid droplet dispersion. However, adenovirus directed expression of FSP27 does not protect against the catecholamine-stimulated lipolysis. These results are consistent with my previous results suggesting lipolysis and lipid droplet dispersion are two separate processes, and also suggest that FSP27 depletion, unlike TNF-α, is not a mechanism of catecholamine-mediated lipolysis. However, I hypothesize that the stabilization, and increase of FSP27 after isoproterenol treatment of adipocytes is a feedback mechanism to restrain the potent lipolytic effect of catecholamines because there is an increase in the rate of lipolysis in response to isoproterenol treatment when the isoproterenol-mediated increase in FSP27 levels is prevented using siRNA.

These studies show that modulation of the cellular levels of FSP27, a rapidly turned-over protein, is an important mechanism by which lipolytic stimuli regulate lipolysis in adipocytes. The lipolytic cytokine, TNF-α, increases lipolysis in adipocytes by decreasing FSP27. On the other hand, catecholamines increase cellular FSP27 by decreasing ubiquitination and degradation, thereby possibly restraining its own potent lipolytic effect.

INTRODUCTION

Storing excess energy for future use during starvation is critical for survival of mammals. Such excess energy is stored in the form of TGs within lipid

droplets present most abundantly in adipocytes, which in turn are accumulated as depots such as subcutaneous and visceral adipose tissue in mice and humans. TGs in lipid droplets are mobilized in starvation to release energy in the forms of glycerol and FFAs by lipase-catalyzed lipolysis, providing fuel to other cell types such as muscle and liver. Previous work on formation of lipid droplets and regulation of lipolysis have elucidated the importance of lipid droplet associated proteins for these processes (256, 259). Based on shared sequence homology, one set of lipid droplet proteins is grouped as the PAT (Perilipin-Adipophilin-TIP47) family of proteins (197). PAT-related proteins are functionally conserved from mammals to lower organisms such as *Drosophila* and *Dictyostelium* (260). In *Drosophila*, two PAT domain proteins are encoded by *Lsdp1* and *Lsd2* genes. Loss-of-function *Lsd2* mutants in *Drosophila* are lean whereas overexpression of *Lsd2* causes obesity (261). In mammals, PAT proteins can be divided into exchangeable TG-associated PAT proteins (EPATs) or constitutively TG-associated PAT proteins (CPATs). EPATs include TIP47, S3-12 and OXPAT/MLDP. These are stably expressed in cytosol and bind nascent TAG droplets. CPATs include perilipin and adipophilin, which are bound to neutral lipid droplets, and are rapidly degraded when dissociated from the lipid droplets (197). Thus, expression of the different sets of lipid droplet proteins contributes to the finely tuned regulation of TG deposition in lipid droplets.

Lipolysis of TG into glycerol and FFAs is also controlled by various physiological stimuli such as β-adrenergic agonists in fasting, and TNF-α secreted by macrophages that infiltrate adipose tissues in obesity. Stimulation of β-adrenergic receptors by the catecholamines epinephrine, norepinephrine and isoproterenol activates adenylyl cyclase, increasing intracellular cAMP levels, and activating cAMP-dependent PKA. This protein kinase catalyzes phosphorylation of HSL as well as perilipin, which can then interact and enhance lipolysis (180, 182-186). TNF-α on the other hand decreases perilipin expression, which also leads to increased lipolysis, albeit with a longer time course and to a lesser extent than the effect of catecholamines. Additionally, preventing the depletion of perilipin by TNF-α by expression of perilipin via an adenovirus vector has been shown to protect against TNF-α mediated lipolysis (174). In addition, adipocytes from perilipin null mice display a higher basal lipolytic rate (215, 216). These mice exhibit an attenuated lipolytic response to β-adrenergic stimulation, reinforcing the importance of perilipin in the mechanism of lipase action to enhance TG hydrolysis.

Recently, our laboratory identified FSP27 as a highly expressed adipocyte protein that also associates with lipid droplets (233) and has a significant homology to domains in perilipin thought to be responsible for triglyceride shielding from lipases and for lipid droplet targeting and anchoring (231).

These findings have been confirmed and extended to show that FSP27 expression is associated with increased lipid droplet size and triglyceride accumulation in adipose and non adipose cell-types (233-235, 249). FSP27 is a member of CIDE (Cell Death Inducing DFF45-like effector) family of proteins that have a common CIDE-N domain in the N-terminus and CIDE-C domain in the C-terminus (230). Thus, the human FSP27 is also denoted CIDEC. FSP27 was first identified as an adipocyte-specific gene (262) and later shown to have homology to 45kDa subunit of DNA fragmentation factor (230). It is highly expressed in white adipose tissue in mice as well as humans, and has been shown to be upregulated during 3T3-L1 adipogenesis (235). Despite having CIDE domains, FSP27 upregulation during adipogenesis does not lead to apoptosis (235). Proteomic analysis of lipid droplet-associated proteins in adipocyte homogenates has also revealed FSP27 to be in the lipid droplet fraction (263). Studies in both cultured cells and intact mice have confirmed that FSP27 depletion causes lipid droplet fragmentation in adipocytes (233, 234), suggesting it is necessary for formation of large unilocular lipid droplets of primary mouse and human adipocytes.

Since siRNA-mediated depletion of FSP27 causes increased lipolysis of lipid droplet TGs in adipocytes, and its overexpression enhances TG deposition in cells, my aim in the present study is to examine its potential role in the

physiological regulation of lipolysis by TNF-α and catecholamines. In order to study the regulation of FSP27 by TNF-α, and catecholamines, first, I studied the regulation of FSP27 in adipocytes in the basal state. The data presented here show that FSP27 is under the transcriptional regulation of PPARγ, the most important transcription factor in adipocytes (231). It is later confirmed that PPARγ2 binds to the PPARγ response elements on FSP27 promoter in adipocytes to drive FSP27 expression (246). FSP27 is also observed to be PPARγ target gene in hepatocytes (264). Furthermore, the studies in this thesis show that FSP27 has extremely rapid turnover with a half-life of less than 15 min. It is ubiquitinated, and degraded via proteasome similar to CIDEA and other lipid droplet proteins such as perilipin and ATGL (209, 223, 238). These results are consistent with the paper published during preparation of this thesis (258). I also report that TNF-α treatment of cultured adipocytes causes FSP27 depletion followed by decreased lipid droplet size, and lipolysis. Conversely, sustained FSP27 expression in adipocytes from a viral expression vector protects against TNF-α action on lipid droplet size, and lipolysis. This study highlights the importance of the lipid droplet protein FSP27 as a key target of TNF-α in the mechanism whereby this cytokine enhances lipolysis, and decreases lipid droplet size in adipocytes.

On the other hand, isoproterenol, a potent lipolytic agent, surprisingly increases FSP27 levels by decreasing FSP27 ubiquitination, and

degradation. The increase in FSP27 levels by isoporterenol is mediated via canonical PKA pathway, and increases in intracellular cAMP. The adenoviral driven expression of FSP27 is able to protect against isoproterenol effect on lipid droplet size but not on lipolysis as we see larger and fewer lipid droplets associated with robust lipolysis when the adipocytes expressing adenoviral FSP27 are treated with isoproterenol. This suggests that catecholamine mediated lipolysis, and change in lipid droplet morphology are distinct processes, and catecholamine leads to lipolysis in adipocytes through a pathway different from TNF-α and FSP27.

This study highlights the importance of the lipid droplet protein FSP27 as a key target of TNF-α and β-adrenergic agonists in the mechanisms whereby these agents modulate lipolysis in adipocytes.

EXPERIMENTAL PROCEDURES

Materials- Affinity purified rabbit polyclonal FSP27 antibody was generated in the lab and also was kindly provided by Drs. Kasuga and Nishino, Kobe University Graduate School of Medicine, Kobe. Polyclonal perilipin antibody was purchased from Fitzgerald (20R-PP004). Polyclonal GFP antibody (2555), monoclonal HA antibody (2367) and monoclonal ATGL antibody (2439) were purchased from Cell Signaling. Monoclonal Ubiquitin antibody

P4D1 (sc-8017) was purchased from santa cruz and Vti1α from BD Transduction Laboratory (611220) Recombinant murine TNF-α (654245), cycloheximide (2397650), and MG-132 (474790) were obtained from Calbiochem. Isoproterenol was purchased from Sigma (15627-56). 8-Bromo-cAMP (CN-115), Forskolin (CN-100), and KT-5720 (EI-199) were obtained from Enzo Life Sciences, Leupeptin, Aprotinin from Roche, and NH4Cl from EMD.

siRNA- All siRNAs were purchased from Dharmacon (Chicago). The siRNA sequences were: Scr: 5'-CAGUCGCGUUUGCGACUGG-3'; FSP27: 5'-CAACUAAGAAGAUCGAUGUUU-3'; ATGL: 5'-GCACAUUUAUCCCGGUGUAUU-3'; PKAca: 5'-GUGGUUUGCCACGACUGACUU-3'; PKAcb: 5'AGAGUUUCUAGCCAAAGCCUU-3'

Cell Culture and siRNA Transfection in 3T3-L1 Adipocytes- 3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50µg/ml penicillin, and 50µg/ml streptomycin (complete DMEM). After they reached quiescence, they were differentiated into adipocytes as previously described (265). Adipocytes that were differentiated for four days were transfected with siRNA duplexes using a Biorad electroporator also described previously (265, 266).

Generation of Adenovirus and Infection of Adipocytes with Adenovirus- The coding sequence of mouse FSP27 with an HA tag on the N-terminal end was cloned into the pAdTrack adenoviral vector in Sal1/BglII restriction enzyme sites in the multiple cloning region. The construct was confirmed by sequencing. The construct was then linearized by Pme1 digestion and transformed into BJ5813 electrocompetent cells (Stratagene) to integrate the construct by homologous recombination into adenoviral backbone as described for the Adeasy system for viral vector generation (267). Empty vector with GFP was used as control. Expression of HA tagged FSP27 and GFP were standardized with titration and confirmed with western blot analysis using FSP27, HA, and GFP antibodies. For infection of adipocytes, control or FSP27 adenovirus was directly added for 24hr to adipocytes on the fourth day after initiation of differentiation. At the end of 24 hrs, the media was changed to fresh complete DMEM and cells were cultured for another 48 hr before harvesting. All experiments were designed such that the cells had been exposed to virus 72 hours prior to harvesting.

TNF-α and Isoproterenol Treatment, and Assay to Measure Glycerol Release- To treat adipocytes with TNF-α or isoproterenol, adipocytes were washed with PBS once. Then 10 ng/ml TNF-α or 10 μM isoproterenol was added to phenol red free high glucose DMEM supplemented with 1% sodium

pyruvate, 1% L-glutamine, 0.1% biotin, 10% fetal bovine serum, 1% of penicillin, and 1% of streptomycin for 16hr or 3hrs respectively. For serum starvation, cells were washed with PBS once, and phenol red free high glucose DMEM supplemented with 1% sodium pyruvate, 1% L-glutamine, 0.1% biotin, and 2% bovine serum albumin was added overnight, and then the cells were treated with TNF-α. After TNF-α or isoproterenol treatment the media was collected and assayed for glycerol content using a commercial colorimetric assay kit from Sigma as per manufacturer's protocol.

FSP27 stability Assay: Mature 3T3-L1 adipocytes were treated with 5μg/ml Leupeptin, 5μg/ml Aprotinin, 10 μM MG132, or 2.5 mM NH_4Cl for 2 hrs, protein harvested with SDS lysis buffer, and analyzed with western blotting. For cycloheximide chase studies, mature adipocytes were washed with PBS and treated with 5μg/ml cycloheximide for 15, 30, and 60 min, then protein harvested with SDS lysis buffer and analyzed with western blotting.

Detection of FSP27 ubiquitination: Mature 3T3-L1 adipocytes were treated with 10μM MG132 alone or in combination with 10ng/ml TNF-α, or 10μM isoproterenol for 3 hr. The cells were washed twice with ice cold PBS and harvested with RIPA buffer containing 0.5% SDS. The lysate was passed through syringe and precleared with Protein A beads for 1 hr. FSP27 was immunoprecipitated with either non-immune rabbit IgG or Rabbit FSP27

antibody from the precleared lysate diluted with lysis buffer to final SDS concentration of 0.1%. The immunoprecipitated protein was analyzed with western blot and probed with ubiquitin, and FSP27 antibody.

Oil Red-O Staining for Lipid Droplets- Adipocytes grown on coverslips were washed three times with cold PBS, fixed in 4% paraformaldehyde for 10 mins, permeabilized and blocked with 0.05% Triton X-100 and 5% fetal bovine serum in PBS for 30 mins. Oil Red-O (0.4%) in isopropyl alcohol solution was mixed with distilled water in the ratio of 60:40 and filtered through 0.45µm filters. This freshly prepared Oil red-O solution was added to permeabilized cells for 30 mins. These cells were then washed three times with ice cold PBS and mounted using Prolong Gold + DAPI mounting medium (Invitrogen), and lipid droplets imaged by confocal microscopy with a X60 Nikon PlanApo objective. All images were identically exposed in any given experiment.

Confocal Microscopy- Images were taken using a Zeiss Axiophot microscope with Hamamatsu digital camera and processed with Metamorph Imaging software (version 6.1). Quantitation of the lipid droplet size was done using Axiovision digital imaging software (version 4.1) by selecting individual lipid droplets or groups of lipid droplets where appropriate.

RNA Isolation and Quantitative Reverse Transcription-PCR- Total RNA from 3T3-L1 adipocytes were isolated using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. cDNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-Rad). Quantitative real time PCR was done using a MyIQ real-time PCR system with iQ SYBR Green mix (Bio-Rad) and specific primers to amplify the genes.

Statistical Analysis- Results are expressed as mean ± S.E. and the significance of the change were assessed using Student's t-test or one-way ANOVA test.

RESULTS

A. Regulation of FSP27

FSP27 is a PPARγ responsive gene - FSP27 is highly expressed in adipocytes that are under the transcriptional control of factor PPARγ. In addition, FSP27 is a lipid droplet protein, and PPARγ promotes lipid accumulation in adipocytes. Therefore, I tested if FSP27 is one of the target genes of PPARγ. 3T3-L1 adipocytes were transfected with PPARγ siRNA on the fourth day of differentiation, and RNA was harvested to measure PPARγ and FSP27 transcripts using real time PCR. Depletion of PPARγ by 90% decreased FSP27 to a similar extent suggesting that FSP27 is a PPARγ

target gene (Fig. 2.1 A). Other studies had also shown that FSP27 mRNA progressively increased during adipocyte differentiation (235). It had later been confirmed by the study that showed PPARγ2 binds to -219/-207 promoter region in FSP27 that contains a PPARγ responsive element, and drives the transcription of FSP27 (246). Furthermore, in hepatocytes also, the expression of FSP27 was markedly decreased in *ob/ob* mice deficient in PPARγ (264). These data show that FSP27 is one of the PPARγ responsive genes.

A.

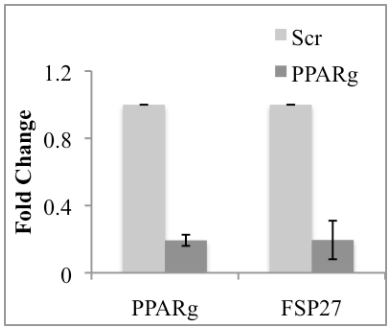


Fig. 2.1. FSP27 is a PPARγ responsive gene. A. Mature 3T3-L1 adipocytes were transfected with Scr or PPARγ siRNA for 72 hr and the harvested RNA was analyzed for PPARγ and FSP27 mRNA using real time PCR. There was about 90% depletion of FSP27 mRNA in parallel to similar depletion in PPARγ mRNA. Data expressed as mean ± SE of three different experiments.

FSP27 has a short half-life and is degraded by proteasome – Previous studies have shown that FSP27 depletion increases lipolysis in adipocytes while FSP27 expression promotes enlarged lipid droplet formation in COS cells and preadipocytes (233, 234). These data suggest that the changes in cytosolic FSP27 levels could modulate lipid metabolism or storage, and could be a mechanism by which various stimuli modulate lipolysis. Therefore, in order to study the role of FSP27 in regulation of lipolysis in response to various lipolytic stimuli, I studied the regulation of FSP27 protein levels. First I determined the stability of FSP27 by treating mature adipocytes with 5μg/ml of cycloheximide to block new protein synthesis and measured intact FSP27 protein. I found that FSP27 is an unusually short-lived protein and has a half-life of less than 15 min (Fig. 2.2 A, B). Addition of the proteasome inhibitor MG132 inhibited the degradation of FSP27 (Fig. 2.2C,D). However, neither the general peptidase inhibitors aprotinin and leupeptin nor the lysosome inhibitor NH_4Cl was able to prevent degradation of FSP27 (Fig. 2.2C,D). Another group also published recently showing similar results that FSP27 is a fast turned-over protein and is degraded via proteasome (258).

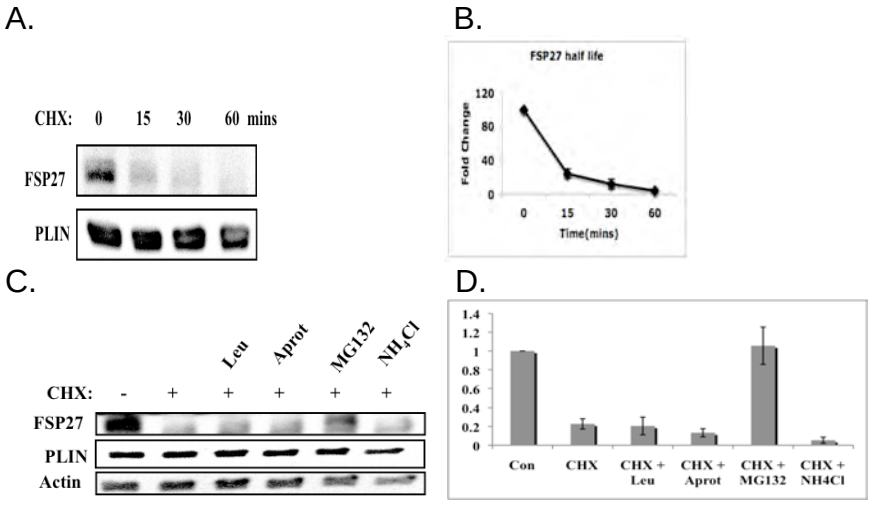


Fig. 2.2. FSP27 has a short half-life and is degraded by proteasome after ubiquitination. A. Representative western blot of adipocyte lysates following 15, 30 and 60 minutes treatment with 5μg/ml of cycloheximide compared to untreated (0) cells shows rapid degradation of FSP27. B. Densitometric measurement of FSP27 protein levels after cycloheximide treatment as in (A). C. Representative western blot of FSP27 shows protection of FSP27 from degradation in lysates from cells treated with the proteasome inhibitor MG132, but not by peptidase inhibitors leupeptin (Leu) or aprotinin (Aprot), or lysosomal inhibitor NH_4Cl. D. Densitometric measurement showing protection of FSP27 by MG132. Data shown representative of six separate experiments.

FSP27 is ubiquitinated to target for proteasomal degradation- It has been well established that the proteins degraded via proteasome are ubiquitinated as a signal for degradation (268). This is also observed in other lipid droplet proteins such as perilipin, ADRP, and one of the CIDE proteins, CIDEA (209, 223, 238). Therefore, I tested whether FSP27 is ubiquitinated for proteasomal degradation. In order to detect ubiquitination of FSP27, mature 3T3-L1 adipocytes were treated with MG132 for 3 hr to block proteasomal degradation of FSP27, and harvested using RIPA buffer containing 0.5% SDS. Equal amounts of protein were taken, diluted with lysis buffer to get final concentration of 0.1% SDS, and FSP27 was immunoprecipiated overnight using anti-FSP27 antibody, then analysed by Western blot anti-ubiquitin antibody and anti-FSP27 antibody. Upon blockade of proteasomal degradation with MG132, multiple bands detectable with anti-ubiquitin antibody as well as FSP27 antibody could be detected specifically in FSP27 immunoprecipitates (Fig. 2.3 B,C). These results suggest that FSP27 protein is rapidly destroyed by ubiquitination and proteasomal degradation, raising the possibility that FSP27 protein levels could be quickly modulated as a mechanism for regulation of lipolysis. These data are consistent with a paper published as we were finalizing our manuscript that also showed FSP27 has a short half life and is ubiquitinated and degraded via the proteasome (258).

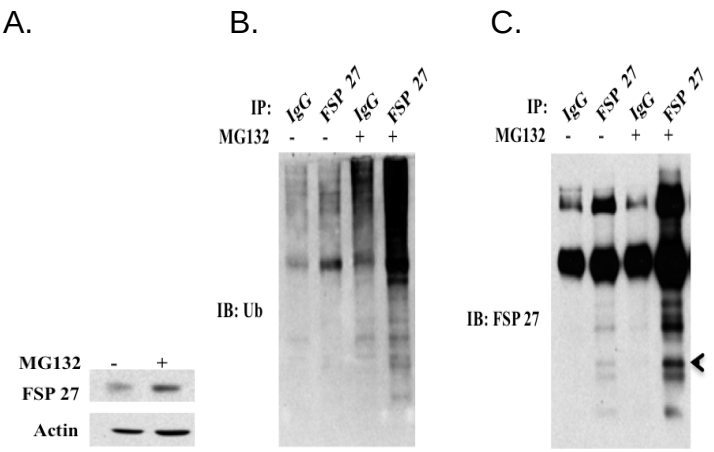


Fig. 2.3. FSP27 is ubiquitinated to target for proteasomal degradation. Mature 3T3-L1 adipocytes were treated with MG132 for 3 hr, and harvested in RIPA buffer containing 0.5% SDS. A. Total lysate of 3T3-L1 adipocytes with or without MG132. B. FSP27 immunoprecipitated with endogenous FSP27 antibody or non-immune rabbit IgG (IgG), and probed with anti-ubiquitin antibody shows increased ubiquitination in presence of MG132. C. FSP27 immunoprecipitated using endogenous FSP27 antibody or non-immune rabbit IgG (IgG), and probed with anti-FSP27 antibody shows polyubiquitinated FSP27. Data shown representative of four separate experiments.

B. Suppression of FSP27 by TNF-α to mediate lipolysis in 3T3-L1 adipocytes

TNF-α decreases FSP27 levels in 3T3-L1 adipocytes - TNF-α is an inflammatory cytokine that has profound effects on adipocytes including stimulation of lipolysis and downregulation of numerous adipocyte-expressed genes. Since FSP27 depletion causes increased lipolysis in adipocytes I sought to determine whether TNF-α alters FSP27 levels when adipocytes are treated with TNF-α at concentrations that stimulates lipolysis. The lowest concentration and shortest duration of TNF-α treatment that stimulates lipolysis optimally was established by treating fully differentiated mature 3T3-L1 adipocytes with various concentrations of TNF-α for different time periods and measuring rates of glycerol release. The strongest effects on lipolysis were obtained when cultured adipocytes were treated with 10 ng/ml TNF-α for 16 hrs. Under these conditions about a 1.5 fold increase in glycerol release from adipocytes was observed in response to the cytokine. Although, this concentration of TNF-α is comparable to the plasma TNF-α level in NOD mice that developed diabetes after treatment with interferon-γ and lipopolysaccharide (269), the local concentration of TNF-α in adipocytes could be higher than circulating plasma levels because of increased TNF-α expression in adipocytes in obesity and inflammation (270). Nevertheless, this TNF-α concentration is likely similar to low grade inflammation associated

with obesity and insulin resistance. Thus, for the subsequent experiments, this treatment condition was used.

After treatment of mature adipocytes with TNF-α, FSP27 and perilipin mRNA were analyzed using quantitative real time PCR. Treating adipocytes with TNF-α significantly decreased the mRNA transcripts of both FSP27 and perilipin (Fig. 2.4 A). Downregulation of FSP27 mRNA after TNF-α treatment was also observed previously with Northern blotting (235), and downregulation of perilipin by the cytokine is consistent with a previous study (174). The effect of TNF-α on the protein levels of FSP27 and perilipin were also analyzed using Western blotting, which showed a significant decrease in FSP27 and perilipin protein levels after TNF-α treatment (Fig 2.4 B, C). Therefore, TNF-α treatment of adipocytes, at the dose stimulating lipolysis, downregulates FSP27 both at mRNA and protein levels.

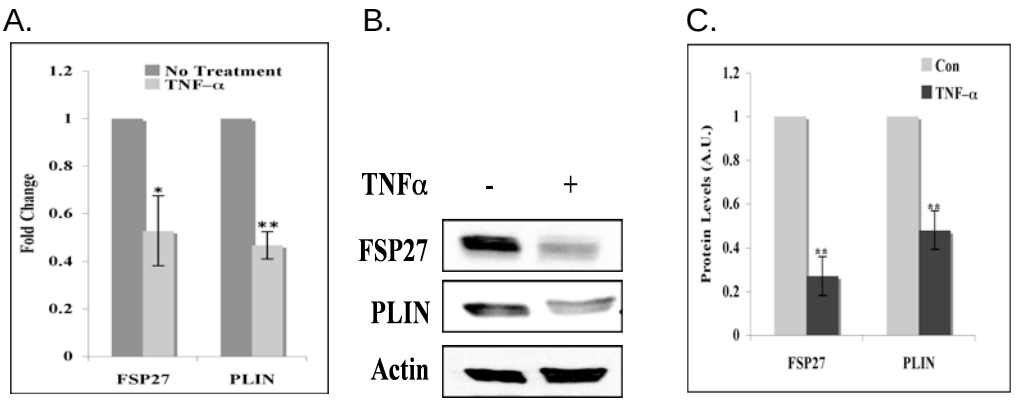
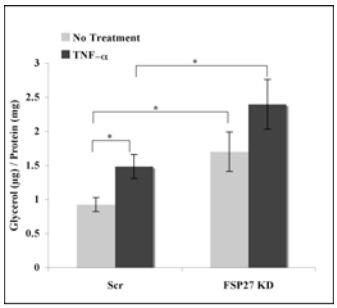


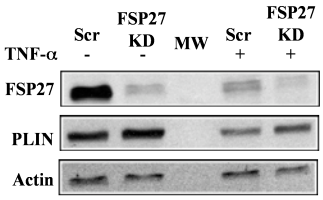
Fig. 2.4. TNF-α decreases FSP27 levels in 3T3-L1 adipocytes. A. RT-PCR showing decreased FSP27 and perilipin (PLIN) mRNA in mature 3T3-L1 adipocytes treated with 10ng/ml TNF-α for 16 hrs. B. Western Blot analysis using FSP27, PLIN, and Actin (loading control) antibodies showing decrease in FSP27 and PLIN protein levels in 3T3-L1 adipocytes treated with 10 ng/ml TNF-α for 16 hrs. C. Densitometry showing decrease in FSP27 and PLIN protein levels after TNF-α treatment. Data expressed as means ± S.E for six different experiments, p-value calculated using Student's t test, p *<0.05, ** < 0.0005.

FSP27 depletion enhances TNF-α-stimulated lipolysis - To determine whether FSP27 protein downregulation by TNF-α plays a role in increased lipolysis, I first measured lipolysis in adipocytes after siRNA-mediated depletion of FSP27 with or without TNF-α treatment (Fig. 2.5). Cultured adipocytes were transfected with either scrambled (Scr) or FSP27 siRNA, and glycerol release was measured in basal and TNF-α-treated cells. Treatment of cells with TNF-a and silencing of FSP27 expression both caused a ~50% increase in glycerol release, while the combination of these treatments resulted in a 2-2.5 fold increase in lipolysis (Fig. 2.5 A). Both FSP27 silencing and TNF-a treatment caused similarly dramatic decreases in FSP27 protein levels (Fig. 2.5 B,C). Under these conditions TNF-α caused a smaller but still significant decrease in perilipin levels as previously reported (174). However, FSP27 depletion by gene silencing did not significantly change perilipin levels although we detected increased glycerol release, suggesting that perilipin is unable to compensate for decreased FSP27 levels in preventing lipolysis under these conditions. Thus, depletion of FSP27 correlates with increased glycerol release, implicating control of FSP27 as a potential mechanism of regulation of lipolytic rate.

A.



B.



C.

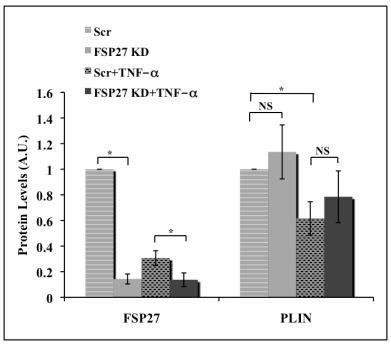


Fig. 2.5. TNF-α treatment of 3T3-L1 adipocytes increases lipolysis that is enhanced by further depletion of FSP27 using siRNA. A. Glycerol released in media measured in 3T3-L1 adipocytes electroporated with scr or FSP27 siRNA, and treated with 10 ng/ml TNF-α for 16 hrs. siRNA mediated depletion of FSP27 in TNF-α treated cells causes maximum increase in glycerol release. B. Western blot analysis using FSP27, perilipin (PLIN), and Actin antibodies showing FSP27 and PLIN protein levels in control and TNF-α treated conditions. C. Densitometric measurement of fig. B showing decrease in FSP27 levels with siRNA and TNF-α treatment. Data expressed as means ± S.E for six different experiments, p-value calculated using ANOVA, p *<0.05, ** < 0.0005

TNF-α mediated decrease in FSP27 precedes the diminution of lipid droplet size and increase in glycerol release – To further address potential regulation of lipolysis by FSP27 I examined the temporal relationships between TNF-α-mediated changes in decreased FSP27 protein levels, lipid droplet morphology and adipocyte lipolytic rates. Mature 3T3-L1 adipocytes were treated with 10ng/ml TNF-α for various times between 30 min and 16 hr as shown in Figure 2.6, and the media collected and assayed for glycerol release. There was a significant increase in lipolysis starting at 2 hr after addition of TNF-α to the adipocytes, but the highest increase in lipolytic rate occurred at 16hr (Fig. 2.6 D). Whereas the FSP27 protein levels started to decrease as early as 30 min after addition of TNF-α treatment, the protein was almost completely undetectable after 2 hr of treatment (Fig. 2.6 E, F). The size of lipid droplets within adipocytes was already reduced after only 30 min of TNF-α treatment and increased numbers of lipid droplets could also be observed at this time (Fig. 2.6 A, B, C). These changes became statistically significant after 1 hr of TNF-α treatment. Thus, the rapid TNF-α-mediated decrease in FSP27 protein preceded formation of numerous smaller lipid droplets. Increased glycerol release follows these events suggesting that the change in lipid droplet morphology that results from loss of FSP27 facilitates subsequent events that increase lipolytic rate.

FIGURE 2.6

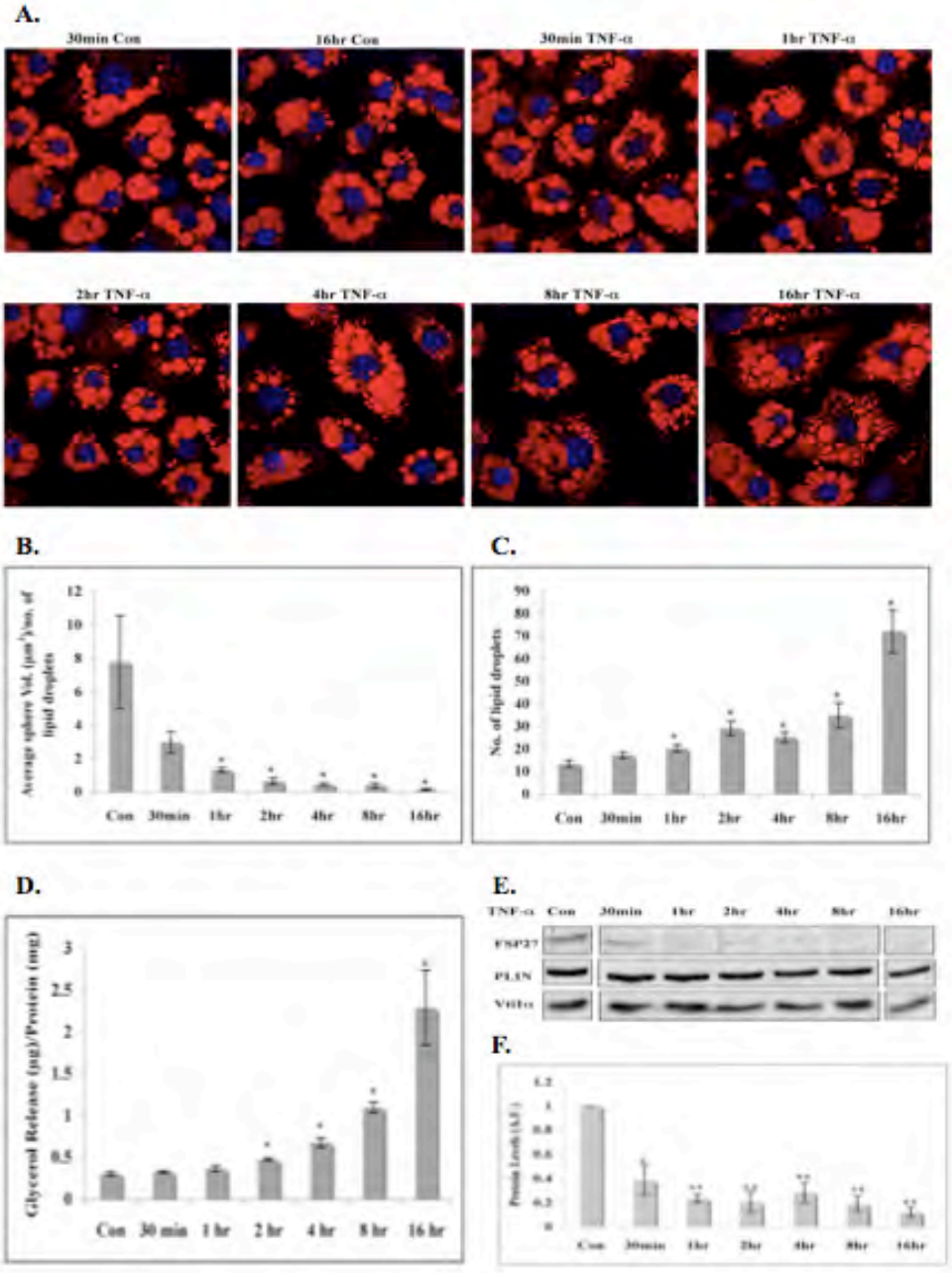

A.
30min Con
16hr Con
30min TNF-α
1hr TNF-α
2hr TNF-α
4hr TNF-α
8hr TNF-α
16hr TNF-α
B.
Average sphere Vol. (μm³)/no. of lipid droplets
Con 30min 1hr 2hr 4hr 8hr 16hr
C.
No. of lipid droplets
Con 30min 1hr 2hr 4hr 8hr 16hr
D.
Glycerol Release (μg)/Protein (mg)
Con 30 min 1 hr 2 hr 4 hr 8 hr 16 hr
E.
TNF-α Con 30min 1hr 2hr 4hr 8hr 16hr
FSP27
PLIN
Vinculin
F.
Con 30min 1hr 2hr 4hr 8hr 16hr

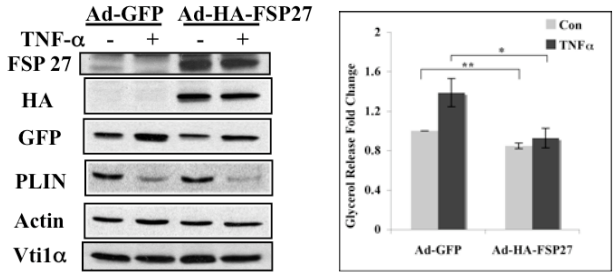
FIGURE 2.6. TNF-α mediated decrease in FSP27 precedes the diminution of lipid droplet size and increase in glycerol release. Mature 3T3-L1 adipocytes were treated with TNF-α for 30 min, 1hr, 2hr, 4hr, 8hr and 16hr. A. Confocal microscope image showing lipid droplets stained with Oil Red-O (red), and nucleus stained with DAPI (blue). Images shown are representative of 90 random fields imaged in three different experiments. B. Quantification of lipid droplet size using MetaMorph imaging software. Data shown is mean ± S.E of representative 10 cells for each condition from different experiments. C. Quantification of numbers of lipid droplets per cell for each time point. D. Glycerol released into media after 10 ng/ml TNF-α treatment for 30mins, 1 hr, 2 hr, 4 hr, 8 hr, and 16 hr. E. Western Blot analysis showing the FSP27 protein after 10 ng/ml TNF-α treatment for 30 min, 1 hr, 2 hr, 4 hr, 8 hr, and 16 hr. F. Densitometry showing decrease of FSP27 protein after TNF-α treatment. Data expressed as mean ± S.E for three different experiments, p-value calculated using ANOVA, p *<0.05, ** < 0.005

Expression of adenoviral FSP27 protects against TNF-α mediated lipolysis and reduced lipid droplet size - To test the hypothesis that the action of TNF-α on adipocyte lipolysis relies on its ability to deplete FSP27 protein levels, we developed a strategy to maintain FSP27 protein levels in adipocytes in the presence of TNF-α. To achieve this, we generated an HA-tagged FSP27 construct incorporated into an adenovirus expression system that also elicits GFP expression from a different promoter, thus maintaining FSP27 levels when the endogenous FSP27 expression is reduced by TNF-α. A virus expressing GFP alone was used to infect control cells. Using these reagents, recombinant adenovirus-directed expression of HA-FSP27 remained constant even when TNF-α treatment depleted endogenous FSP27 (Fig. 2.7 A). Under these conditions the expected decrease in perilipin protein level in response to the cytokine was also observed. In spite of this, adenovirus-mediated expression of HA-FSP27 in cultured adipocytes blocked the increase in lipolysis caused by TNF-α (Fig. 2.7 B). There was also a small but significant decrease in lipolysis in control adipocytes that were not treated with TNF-α.

Both TNF-α treatment, and FSP27 depletion in cultured adipocytes have been shown to cause a time dependent change in the morphology of lipid droplets such that they are smaller in size and increased in number (197, 233). Therefore, we wanted to test whether high FSP27 expression could

prevent such apparent lipid droplet morphology change upon TNF-α treatment. Control or HA-FSP27 adenovirus were added to differentiated adipocytes seeded on coverslips, and treated with 10 ng/ml TNF-α. The cells were then stained with Oil Red-O to visualize lipid droplets. Treatment of the cultured adipocytes with TNF-α for 16 hr caused formation of smaller and more abundant lipid droplets compared to control cells, as expected. However, TNF-α treatment of adipocytes expressing HA-FSP27 maintained larger, fewer lipid droplets compared to cells expressing GFP alone or uninfected adipocytes in the same field (Fig. 2.7 C). These results demonstrate that sustained FSP27 expression can block the effects of TNF-α on both lipid droplet morphology and lipolysis even though perilipin levels are diminished. This suggests that depletion of FSP27 is part of the mechanism by which TNF-α increases lipolysis in adipocytes.

A. B.



C.

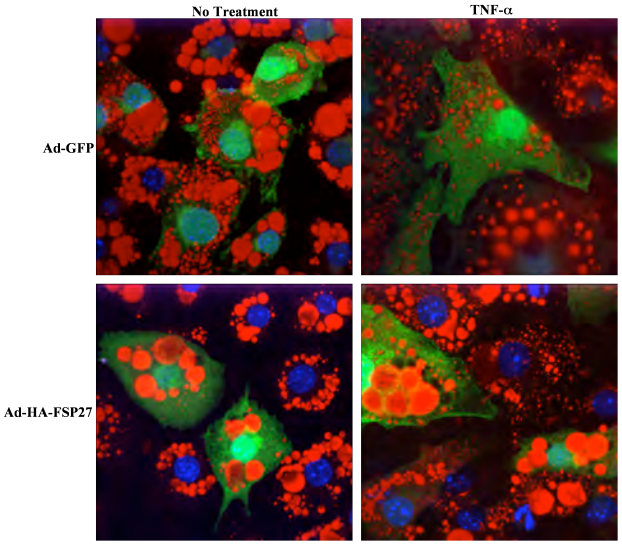


Fig. 2.7. Expression of adenoviral FSP27 protects against TNF-α mediated lipolysis and reduced lipid droplet size. 3T3-L1 adipocytes were infected with control virus (Ad-GFP) or HA-FSP27 expressing virus (Ad-HA-FSP27) on the fourth day of differentiation. On the fifth day, cells were serum starved overnight, then treated with 10 ng/ml TNF-α for 24 hrs and analyzed for protein, glycerol release and lipid droplet morphology. A. Western blot analysis using FSP27, HA, GFP, perilipin (PLIN), Actin, and Vti1α antibodies showing maintenance of HA tagged FSP27 protein levels even after treatment of TNF-α. Actin is the loading control for HA, GFP, and PLIN, and Vti1α is the loading control for FSP27. B. Glycerol released into the media in 24 hr with or without TNF-α treatment. C. Confocal microscope image showing lipid droplets stained with Oil Red-O (red), nucleus stained with DAPI (blue), green representing GFP expression in 3T3-L1 adipocytes expressing Ad-GFP or Ad-HA-FSP27 in presence or absence of TNF-α. Data expressed as mean ± S.E for five individual experiments, p-value calculated using ANOVA, p *<0.05, ** < 0.0005

FSP27 depletion and TNF-α both require ATGL to regulate lipolysis - Mouse adipocytes contain multiple lipases including HSL and ATGL that participate in mediating basal and stimulated lipolysis (184, 190-193). Consistent with the concept that these lipases are required for lipolysis, the sizes of lipid droplets were reported to be larger in ATGL- or HSL-depleted adipocytes (184, 189, 271). Thus, we examined whether the lipase ATGL is required to observe the enhanced lipolysis caused by FSP27 depletion and TNF-α treatment. We used siRNA to deplete the lipase ATGL in 3T3-L1 adipocytes and assayed glycerol release. As shown in Fig. 2.8 A, ATGL depletion by siRNA prevented stimulation of lipolysis by TNF-α treatment or FSP27 silencing. Isoproterenol was also not able to enhance lipolysis in the absence of ATGL (Fig. 2.8 B) as expected and reported previously (184, 190-192). These results strongly suggest that TNF-α and FSP27 regulate adipocyte lipolysis through the canonical lipases regulated by catecholamines, which includes TG hydrolysis catalyzed by the lipase ATGL.

A. B.

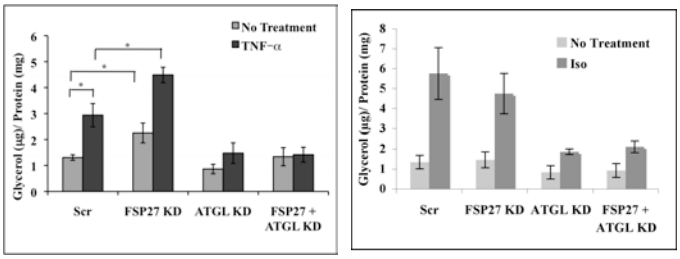


Fig. 2.8. FSP27 depletion and TNF-α both require ATGL to mediate lipolysis. 3T3-L1 adipocytes were electroporated with Scr, FSP27, ATGL, or FSP27 and ATGL siRNA on the fourth day of differentiation. On the sixth day cells were treated with 10 ng/ml TNF-α for 16 hr. A. Glycerol release in media after 16 hrs of 10 ng/ml TNF-α treatment. B. Glycerol release in media after 3 hr of 10 µM Isoporterenol treatment. Data shown is mean± S.E of five individual experiments, p-value calculated using ANOVA, p *<0.05. The attenuation of glycerol release after TNF-α treatment under ATGL KD and/or FSP KD is also significant (p *<0.05).

C. Stabilization of FSP27 by isoproterenol and its effect on 3T3-L1 adipocyte lipolysis

Isoproterenol stabilizes FSP27 by delaying its degradation in 3T3-L1 adipocytes – We also tested the role of FSP27 in β-adrenergic receptor-stimulated lipolysis, which is a normal physiological pathway of starvation-induced lipolysis in adipocytes. First we tested the effect of the β-adrenergic receptor agonist isoproterenol on FSP27 levels. Mature 3T3-L1 adipocytes were treated with 10μM isoproterenol for 3 hr, and protein lysate were harvested and analyzed with western blotting. Surprisingly, treatment of mature adipocytes with 10μM isoproterenol for 3 hr increased FSP27 protein levels in a time dependent manner without significant effects on mRNA levels (Fig. 2.9 A,B,E). Since FSP27 is a short lived protein, the effects of isoproterenol treatment on degradation of FSP27 in adipocytes was determined by treating with 10 μM isoproterenol for 3 hrs, and then treating with cycloheximide to block new protein synthesis. We observed delayed degradation of FSP27 after isoproterenol treatment, increasing its half-life from about 15 minutes to more than 1 hour (Fig. 2.9 C, D), consistent with the data published during preparation of this thesis (258).

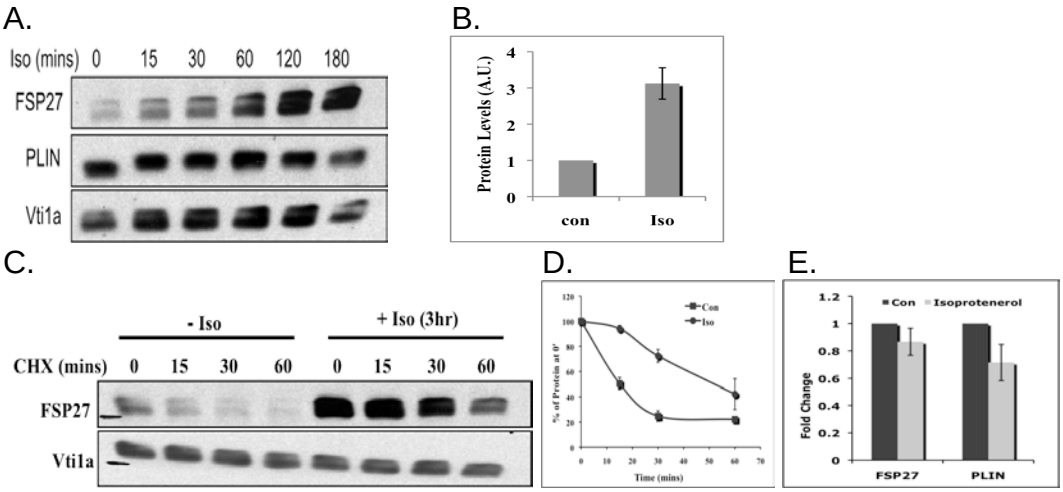


Fig. 2.9. Isoproterenol stabilizes FSP27 by delaying its degradation in 3T3-L1 adipocytes. A. Mature adipocytes treated with 10μM isoproterenol (Iso) for 15, 30, 60, 120 or 180 minutes shows increased FSP27 protein level with isoproterenol treatment compared to the control (0). B. Densitometry showing increased FSP27 protein level after 180mins of 10μM isoproterenol treatment of mature adipocytes. C. Mature adipocytes treated with 10μM isoproterenol for 3 hr, pulsed with 5μg/ml cycloheximide and chased for 15, 30 and 60 minutes show delayed degradation of FSP27 in presence of isoproterenol compared to control. D. Densitometry of FSP27 protein levels show delayed FSP27 degradation in presence of isoproterenol. E. Mature adipocytes treated with 10μM isoproterenol were harvested for RNA and analysed for FSP27 mRNA shows no change in FSP27 transcript with isoproterenol treatment. Data expressed as mean ± S.E for six individual experiments.

Ubiquitination of FSP27 in response to TNF-α and isoproterenol- As isoproterenol upregulated FSP27 protein by delaying its degradation, I hypothesized that isoproterenol would decrease FSP27 ubiquitination. Ubiquitination of FSP27 might also be regulated in response to TNF-α, which decreases FSP27 levels. Mature adipocytes were treated with the proteasome inhibitor MG132 alone or in combination with isoproterenol or TNF-α for 3 hrs, then harvested in RIPA lysis buffer containing 0.5% SDS and MG132. Equal amounts of protein lysate were diluted in lysis buffer to the final SDS concentration of 0.1%, and immunoprecipitated using anti-FSP27 antibody or rabbit non-immune IgG, then FSP27 and ubiquitinated proteins were detected in FSP27 immunoprecipitates. Analysis of FSP27 using ubiquitin antibody showed decreased ubiquitination of FSP27 in response to isoproterenol (Fig. 2.10 B, C). Conversely ubiquitination of FSP27 in response to TNF-α was similar to the MG132 only treatment. Thus, the observed stabilization of FSP27 protein in response to isoproterenol treatment correlates with reduced ubiquitin conjugation to FSP27.

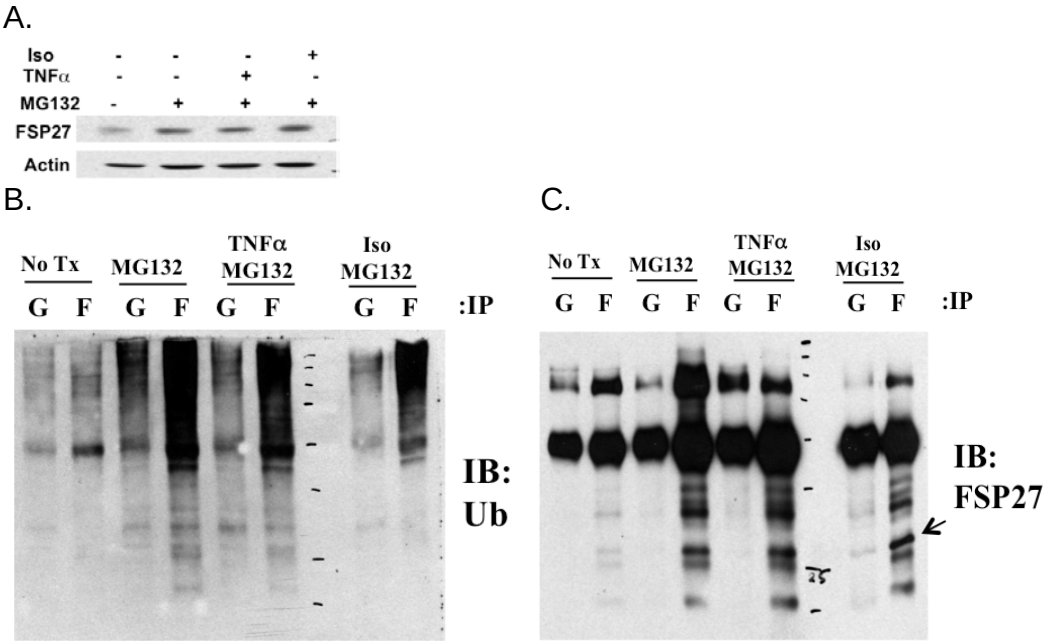


Fig. 2.10. Ubiquitination of FSP27 in response to TNF-α and isoproterenol. Mature adipocytes were treated with MG132 alone or with TNF-α or isoproterenol, FSP27 immunoprecipitated using FSP27 antibody (F) or non immune rabbit IgG (G), and probed with anti-ubiquitin (Ub) and anti-FSP27 antibody. A. Western blot showing total lysate of adipocytes treated with MG132 alone or with TNF-α or isoproterenol. B. FSP27 immunoprecipitated with FSP27 antibody and probed with anti-ubiquitin antibody. C. FSP27 immunoprecipitated with FSP27 and probed with anti-FSP27 antibody. Data shown representative of four separate experiments.

Isoproterenol stabilizes FSP27 through PKA pathway – Because β-adrenergic receptor stimulation increases cAMP levels and activates PKA, we next tested whether isoproterenol-mediated stabilization of FSP27 is via this same canonical PKA pathway. Depletion of PKA using siRNA decreased basal FSP27 level and inhibited upregulation of FSP27 after isoproterenol treatment (Fig. 2.11 A). Effectiveness of the PKA knockdown was evidenced by lack of perilipin phosphorylation (detected as a shifted band in isoproterenol treated cells) upon PKA silencing (Fig. 2.11 A). FSP27 upregulation after isoproterenol treatment was inhibited also by PKA inhibitor KT5720, while the effect of isoproterenol on FSP27 was mimicked by either the adenylate cyclase activator forskolin, or 8-bromo-cAMP, a soluble cAMP analogue, in combination with IBMX, a phosphodiesterase inhibitor (Fig. 2.11 B). Therefore, β-adrenergic stimulation signals via increased intracellular cAMP levels and PKA activation to upregulate FSP27 protein by delaying its degradation.

In next set of experiments I tested whether FSP27 is phosphorylated by PKA in response to isoproterenol. HA tagged FSP27 was expressed in 3T3-L1 adipocytes using adenovirus, and treated with 10 μM isoproterenol for 3 hr. Protein lysates were harvested using RIPA lysis buffer containing 0.5% SDS. Equal amounts of protein were taken, diluted with lysis buffer to final SDS concentration of 0.1%, and was used to immunoprecipitate HA-FSP27 using

anti-HA antibody. No phosphorylated protein was detected in the immunoprecipitates when probed with anti PKA substrate antibody. The efficiency of immunoprecipitation was established by detection of HA-FSP27 when probed with anti-HA antibody (Fig. 2.11 E). Although these results suggest that FSP27 is not phosphorylated in response to isopropterenol treatment, its confirmation requires further investigations.

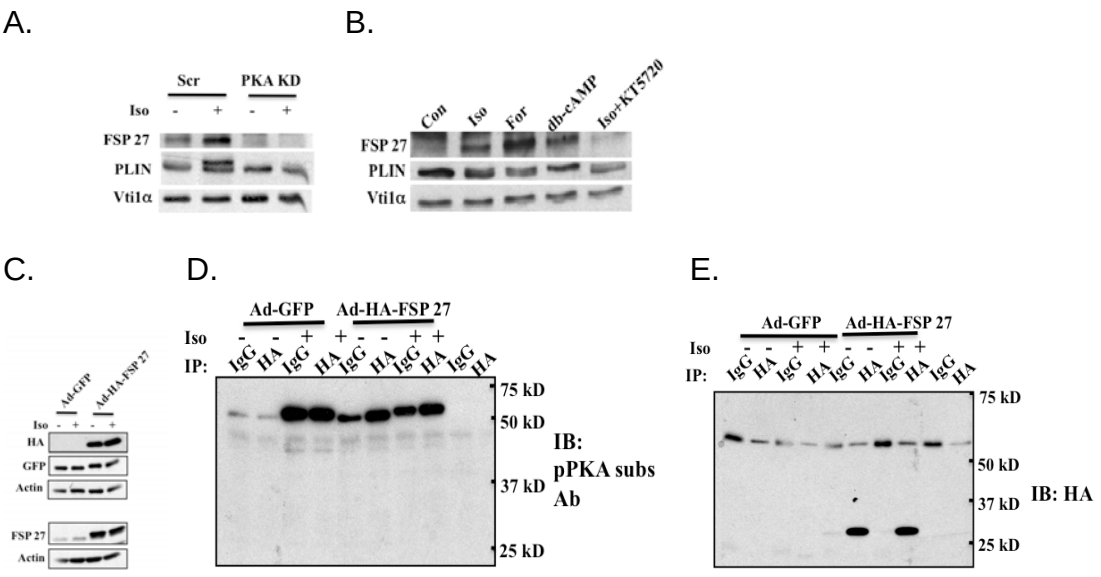


Fig. 2.11. Isoproterenol stabilizes FSP27 through PKA pathway. A. Adipocytes with transfected with either Scr or PKA siRNA and treated with or without 10μM isoproterenol (Iso). The effect of isoproterenol on FSP27 is abrogated when PKA is knocked down. The absence of perilipin (PLIN) phosporylation despite isoproterenol treatment in adipocytes treated with PKA siRNA is used as the positive control. B. Mature adipocytes were treated with 10μM isoproterenol, 5μM Forskolin (For), 1mM 8-bromo-cAMP (BcAMP)+ IBMX or 80 μM KT5720 + 10μM isoproterenol. The effect of isoproterenol on FSP27 is mimicked by Forskolin, BcAMP, and inhibited by KT5720. C. Western Blot of total lysate from 3T3-L1 adipoctes infected with control virus (Ad-GFP) or HA-FSP27 adenovirus (Ad-HA-FSP27) in presence or absence of isoproterenol. D. FSP27 mmunoprecipitated with anti HA antibody or non-immune IgG from Ad-GFP or Ad-HA-FSP27 expressing 3T3-L1 adipocytes in presence or absence of isoproterenol, and probed with anti-PKA substrate antibody does not show any phophorylated band that may correspond to FSP27. E. FSP27 mmunoprecipitated with anti HA antibody or non-immune IgG from Ad-GFP or Ad-HA-FSP27 expressing 3T3-L1 adipocytes in presence or absence of isoproterenol and probed with anti-HA antibody show the immunoprecipitated HA-FSP27.

Isoproterenol-mediated up regulation of FSP27 is independent of isoproterenol-mediated lipolysis – A recently published study has shown that the up regulation of FSP27 after isoproterenol treatment is dependent on fatty acid reesterification, and depletion of diacyl glycerol transferase (DGAT), an enzyme involved in reesterification, prevented FSP27 up regulation in response to isoproterenol (258). Thus, I wanted to test whether isoproterenol mediated lipolysis is an essential event that releases FFAs, which during reesterification stabilizes FSP27. In order to examine this, I blocked lipolysis in 3T3-L1 adipocytes by silencing ATGL, the lipase necessary for stimulated lipolysis. 3T3-L1 adipocytes were transfected with Scr or ATGL siRNA using a BioRad electroporator. After 72 hr of transfection, adipocytes were treated with 10 μM isoproterenol, and media was collected to assay glycerol release. The decrease in glycerol release observed in ATGL depleted adipocytes was consistent with previous results reporting ATGL was required for catecholamine mediated lipolysis (184, 190-192). In addition, significant up regulation of FSP27 after isoproterenol treatment was observed in adipocytes even when lipolysis was blunted in ATGL depleted condition (Fig. 2.12 B, C). Therefore, these results suggest that catecholmine-mediated lipolysis is not required for FSP27 stabilization after catecholamine treatment of adipocytes.

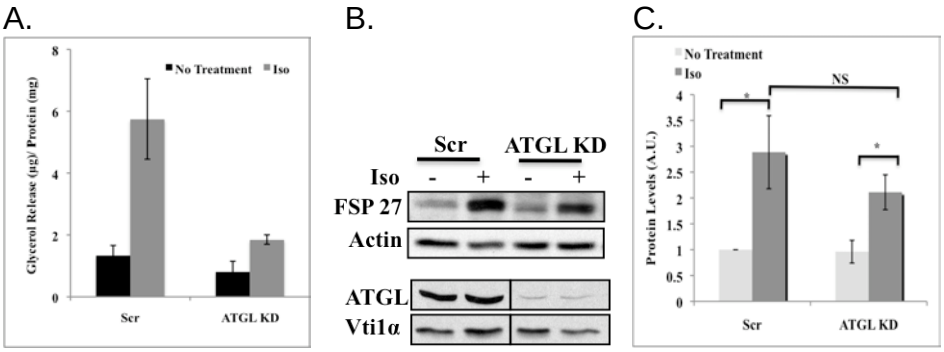


Fig. 2.12. Isoproterenol-mediated up regulation of FSP27 is independent of isoproterenol-mediated lipolysis. 3T3-L1 adipocytes transfected with Scr or ATGL siRNA, and then treated with 10 µM isoproterenol (Iso) for 3 hr. A. Glycerol released in media showing decreased lipolysis in ATGL depleted adipocytes. B. Western blot analysis showing decreased ATGL in ATGL depleted cells whereas FSP27 was upregulated in both Scr and ATGL siRNA treated adipocytes in response to isoproterenol. C. Densitometry of western analysis in fig. B showing significant increases in FSP27 in response to isoproterenol despite decreased ATGL and lipolysis. Data expressed as mean ± S.E for three different experiments, p-value calculated using ANOVA, p *<0.05.

Expression of adenoviral FSP27 protects against Isoproterenol mediated lipid droplet size diminution but not against lipolysis – The up regulation of FSP27, a protein with lipid droplet protective function, upon treatment with potent lipolytic agent, catecholamine was unexpected and surprising. This observation led to the hypothesis that the up regulation of FSP27 upon isoproterenol treatment could be a feedback mechanism to dampen excessive lipolysis after isoproterenol treatment. Therefore, I tested if expression of HA-FSP27 to a higher level using adenovirus could attenuate effects of isoproterenol on lipolysis and lipid droplet size diminution. 3T3-L1 adipocytes were infected with control adenovirus or HA-FSP27 adenovirus for 72 hr as described previously, and treated with 10μM isoproterenol for 3hr. Using adenovirus, we were able to express FSP27 in excess of isoproterenol mediated up regulation of FSP27 (Fig. 2.13 A). When adipocytes expressing HA-FSP27 were treated with 10μM isoproterenol for 3hrs, the normal sharp increase in lipolysis was observed (Fig. 2.13 B). Since isoproterenol upregulated FSP27, it was possible that the protective effective of FSP27 on lipolysis was already saturated, and thus the adenovirus expressed FSP27 did not have an additional protective effect. Thus, I further tested whether there was any difference in the rate of lipolysis mediated by catecholamines in absence of FSP27. FSP27 was depleted in 3T3-L1 adipocytes using siRNA, treated with 10μM isoproterenol for 3 hr, and the rate of glycerol

release in the media was measured using colorimetric assay at the end of 1hr and 3hr. A previous study reported decreased responsiveness to isoproterenol after FSP27 knock-down using siRNA in adipocytes (272) but I observed robust increase in glycerol release after isoproterenol treatment even when FSP27 was depleted using siRNA. Furthermore, the difference in the rate of glycerol release in response to isoproterenol in adipocytes treated with FSP27 compared to Scr siRNA was significantly higher at 3hr compared to 1 hr (Fig. 2.13 C,D). These data suggested that the upregulation of FSP27 after isoproterenol treatment could be an intrinsic mechanism to restrain the potent lipolytic effect of isoproterenol, as the rate of lipolysis was significantly increased if the upregulation of FSP27 by isoproterenol was prevented using siRNA. The data also showed that isoproterenol mediated lipolysis involved a mechanism other than depletion of FSP27 and was different from the mechanism through which TNF-α mediated lipolysis.

My previous results with TNF-α treatment of adipocytes have shown that lipolysis and lipid droplet size diminution are two separate processes. Thus, it was possible that the increased expression of FSP27 from adenovirus might have a protective effect on isoproterenol mediated lipid droplet dispersion despite having no effect on lipolysis. 3T3-L1 adipocytes, infected with adenovirus expressing HA tagged FSP27, were treated with 10 μM isoporterenol for 3 hr. The lipid droplets were stained with oil red-O and

imaged with confocal microscope as described in experimental procedures. Interestingly, HA-FSP27 expression from adevnovirus was able to protect against isoproterenol mediated lipid droplet size diminution. Isoprotenerol caused diminution of lipid droplet size in cells expressing GFP alone, or uninfected cells while those cells expressing FSP27 showed persistent larger lipid droplets even in presence of isoproterenol (Fig. 2.13 D). Taken together, these data indicate that lipolysis and lipid droplet morphology change in response to isoproterenol are two distinct processes, supporting previous observations (183, 218), and FSP27 has a role in formation of larger lipid droplets independent of the lipolysis.

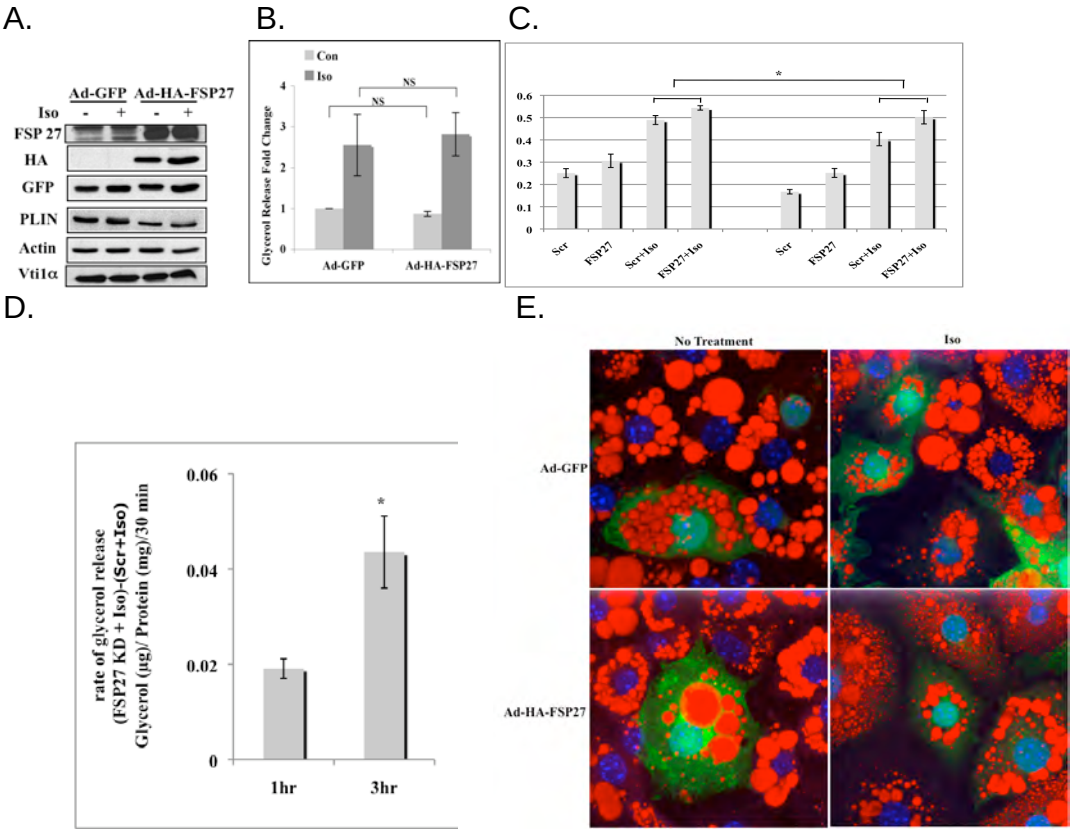


Fig. 2.13. Expression of adenoviral FSP27 protects against Isoproterenol mediated lipid droplet size diminution but not against lipolysis. 3T3-L1 adipocytes were infected control virus (Ad-GFP) or HA-FSP27 expressing virus (Ad-HA-FSP27) on the fourth day of differentiation. On the seventh day, cells were treated with 10µM isoproterenol (Iso) for 3 hrs, and analyzed for protein, glycerol release and lipid droplet morphology. A. Western blot analysis using FSP27, HA, GFP, perilipin (PLIN), Actin, and Vti1α antibodies. Actin is the loading control for HA, GFP, and PLIN, and Vti1α is the loading control for FSP27. B. Glycerol released into the media in 3 hr from adipocytes infected with Ad-GFP or Ad-HA-FSP27, with or without isoproterenol treatment. C. Glycerol released from 3T3-L1 adipocytes treated with Scr or FSP27 siRNA. D. Difference in the rate of glycerol release in response to isoproterenol from 3T3-L1 adipocytes treated with Scr or FSP27 siRNA shows increased rate of lipolysis at 3hr compared to 1hr. E. Confocal microscope image showing lipid droplets stained with Oil Red-O (red), nucleus stained with DAPI (blue), green representing GFP expression in 3T3-L1 adipocytes expressing Ad-GFP or Ad-HA-FSP27 in presence or absence of isoproterenol. Expressed as mean ± S.E for three individual experiments, p-value calculated using ANOVA for fig. B, and using t-test for fig. C, p *<0.05 NS; not significant.

DISCUSSION

The present work as well as another recent study (258) show that FSP27 has a very short half-life, and that its rapid turnover is due to ubiquitination and degradation via the proteasome (Fig. 1). These characteristics are similar to those of other lipid droplet proteins such as perilipin, ADRP and another CIDE protein: CIDEA (209, 223, 238). Recent parallel studies also show that three lysine residues in the C-terminal region of FSP27 – K224, K226 and K236 are critical for ubiquitination and therefore the stability of FSP27 protein (258). Overexpression of mutant FSP27 lacking these three lysines stabilized exogenous and endogenous FSP27, and increased lipid droplet storage in adipocytes. Our results advance this finding by demonstrating that acute regulation of FSP27 levels is one of the mechanisms by which external stimuli such as TNF-α modulate lipolysis (Figures 2, 3, 5).

The cytokine TNF-α has been reported to affect rates of lipolysis in adipocytes at multiple levels, including enhancing cAMP-dependent signaling pathways as well as altering amounts and activities of triglyceride lipases and other lipid droplet associated proteins (166, 176, 256, 273). Here we show that TNF-α causes rapid and dramatic depletion of the lipid droplet protein FSP27 (Fig. 2.4). This depletion precedes the decreased volume of lipid droplets and increased lipolysis (Fig. 2.6). Remarkably, restoration of FSP27

protein levels by adenovirus-mediated expression in TNF-α-treated cells is sufficient to block both the droplet diminution and increased lipolysis induced by TNF-α treatment (Fig. 2.7). This result suggests that downregulation of FSP27 is an essential and early step in the sequence of TNF-α-triggered events that leads to increased triglyceride turnover in adipocytes.

The data presented here show a remarkable, near complete inhibition of FSP27 protein expression by TNF-α in cultured adipocytes. We also observe a significant decrease in FSP27 mRNA after TNF-α treatment (Fig. 2.4 A) consistent with the results previously shown (235). Thus the decrease in FSP27 protein level could be primarily due to the decrease in the FSP27 transcript levels, although we have not ruled out other mechanisms affecting the protein itself. A previous study has shown that TNF-α decreases the expression of a transcription factor, C/EBPα, corresponding to decreased activity of a FSP27 promoter-CAT reporter construct in cultured adipocytes. Thus, downregulation of FSP27 transcripts by TNF-α could be partly due to a decrease in C/EBPα levels (235, 262, 274). Our results and other show that transcription of FSP27 is PPARγ dependent in adipocytes (Fig. 2.1 A, (246)), and in hepatocytes (264) and the effect of TNF-α on FSP27 transcription could be due to the previously observed decrease in PPARγ protein levels in TNF-α-treated adipocytes (275, 276). TNF-α has also been reported to regulate lipolysis in 3T3-L1 adipocytes through MAP kinases (277), however

inhibition of these kinases was not able to block the depletion of FSP27 transcripts (235). The detailed molecular mechanisms whereby TNF-α downregulates FSP27 mRNA and protein is an important topic for future investigations.

Based on previous work cited above, the depletion of FSP27 by TNF-α action could be one mechanism acting in parallel with other mechanisms by which TNF-α stimulates lipolysis in adipocytes. For example, we observed that when FSP27 is depleted using siRNA and the cells are then treated with TNF-α, the increase in glycerol release is greater than FSP27 depletion or TNF-α treatment alone (Fig. 2.5 A). However, this also correlates with lower levels of FSP27 protein after FSP27 silencing with siRNA plus TNF-α treatment compared to either treatment alone (Fig. 2.5 B, C). Importantly, there was no significant change in perilipin expression under these conditions. These data not only support the role of FSP27 in TNF-α stimulated lipolysis, but also indicate that the total lipolysis correlates with the level of FSP27 present in adipocytes. The key role of FSP27 in TNF-α stimulated lipolysis is further supported by the ability of the FSP27 to block TNF-α-stimulated lipolysis in 3T3-L1 adipocytes when there is sustained FSP27 expression using adenovirus infection (Fig. 2.7 B). Consistent with the protection against lipolysis, the phenotype of the lipid droplets with FSP27 expression was large and few in number as opposed to smaller, numerous droplets occurring in

response to TNF-α treatment in control cells (Fig. 2.7 C). Again, under these conditions, the effect of sustained expression of FSP27 on TNF-α mediated lipolysis and lipid droplet phenotypic change occurred despite depletion of perilipin (Fig. 2.7 A, B) suggesting that the role of FSP27 is independent of perilipin. Therefore, our data imply that FSP27 depletion is an important mechanism for TNF-α action on lipolysis under the conditions of our experiments. However, it remains to be studied how FSP27 confers protection against TNF-α effects on lipolysis, and lipid droplet size reduction. It can be speculated that FSP27 may form a protective barrier around lipid droplets, similar to perilipin, and prevent the access of lipases to the TG core.

The role of FSP27 in lipolysis mediated by β-adrenergic receptor activation is different than its role in TNF-α mediated lipolysis. β-adrenergic receptor activation surprisingly increases FSP27 level in a time dependent manner, which contrasts with the inhibitory effects of TNF-α described above (Fig.2.9 A,B). The increase in FSP27 is due to decreased ubiquitination and degradation (Fig. 2.9 C, D, Fig. 2.10 B, C). We also show that β-adrenergic receptor activation signals through canonical PKA pathway and increase in intracellular c-AMP, as addition of Forskolin and soluble B-cAMP mimics the effect whereas PKA inhibition by KT5720 or PKA depletion using siRNA abrogates the effect (Fig.2.11 A, B). However, FSP27 itself doesn't get phophorylated (Fig. 2.11 D, E) suggesting involvement of other mediator

protein(s) that get phophorylated in response to catecholamine and stabilizes FSP27. A recent study shows that oleic acid treatment of adipocytes also increases FSP27 level in a time-dependent manner, and FSP27 stabilization after isoproterenol is dependent upon triacylglycerol synthesis (258). The authors in this study hypothesized that isoproterenol mediated lipolysis is required to provide FFA for esterification, and the process of FFA esterification stabilizes FSP27. However, our results show that FSP27 stabilization occurs independent of isoproterenol mediated lipolysis. It has also been shown in the same study that oleic acid treatment of adipocytes stabilized FSP27 and has been postulated that the stabilization of FSP27 after oleic acide treatment is because of TG synthesis because depletion of an enzyme required for TG synthesis abrogated the stabilization of FSP27. Oleic acid also increases expression of another lipid droplet protein ADRP via transcription factor AP1 and a PPAR response element (278). Thus, the increase in FSP27 after oleic acid treatment may involve similar mechanisms because the FSP27 promoter also has a PPAR response element. Therefore, the mechanism that upregulates FSP27 after oleic acid treatment could be different from β-adrenergic pathway, which has no effect on transcription.

Nonetheless, the increase in FSP27 after a lipolytic stimuli is counterintuitive, and adenovirus mediated expression of FSP27 does not reduce the robust

lipolytic effect of isoproterenol (Fig. 2.13 B). However, it is possible that the adenovirus-mediated expression of FSP27 does not have an effect against isoproterenol mediated lipolysis because the FSP27 expression and function is already at the maximum level. On the other hand, when FSP27 is depleted using siRNA and the adipocytes treated with isoproterenol, there is an increased rate of lipolysis at 3hr, correlating with the difference in cellular FSP27 levels between Scr and FSP27 siRNA treated adipocytes (Fig. 2.13 C). A previous study showed impaired isoproterenol-mediated lipolysis when FSP27 was depleted in 3T3-L1 adipocytes (272), however the data was presented as a ratio of NEFA released in isoproterenol stimulated condition to NEFA released in basal condition, and the decrease in the ratio seen in FSP27 silenced condition could be due to increased basal NEFA release as a result of FSP27 silencing. I observed similar increases in glycerol release after isoproterenol treatment in FSP27 silenced cells and control cells. Furthermore, our results show depletion of FSP27 is not a necessary step for isoproterenol mediated lipolysis, however FSP27 depletion using siRNA increases the rate of lipolysis in response to isoproterenol. Thus, I hypothesize that up regulation of FSP27 protein is a feedback mechanism to restrain excessive lipolysis after strong lipolytic stimuli such as catecholamines.

The data here also suggest that lipolysis and lipid droplet size diminution in response to lipolytic agents are different processes. The decrease in lipid droplet size precedes the detectable release of glycerol in adipocytes treated with TNF-α (Fig. 2.6 A, B, C, D). In addition, adenoviral FSP27 expression protects against both lipid droplet size decrease and lipolysis in response to TNF-α. Furthermore, FSP27 appears to be a point of divergence between TNF-α and β-adrenergic agonist-stimulated lipolysis since expression of adenovirus directed FSP27 does not block increased lipolysis induced by isoproterenol treatment of adipocytes. However, adenovirus directed overexpression of FSP27 is able to protect against isoproterenol mediated lipid droplet reduction. Thus, our study establishes lipolysis and lipid droplet morphology change as distinct processes.

Although FSP27 shares structural and functional similarities with perilipin, there is evidence for divergent and non-redundant roles of these two lipid droplet proteins. Structurally, FSP27 was noted to contain domains homologous to triacylglycerol shielding and targeting domains in perilipin (279). The phenotypes of the null mice for both proteins include resistance to diet induced obesity (215, 216, 234), however perilipin null mice are glucose intolerant whereas FSP27 null mice have normal glucose tolerance. These data suggest divergence in the functions of the two proteins in whole body metabolism. Both perilipin and FSP27 can be downregulated by TNF-α,

however under our experimental conditions siRNA-mediated knockdown of FSP27 led to increased lipolysis despite no significant change in perilipin level (Fig. 2.5 A, B, C). In addition, sustained expression of FSP27 using adenovirus to overcome TNF-α effects on FSP27 was able to protect against the lipolytic effect of TNF-α in spite of the depletion of perilipin. Taken together, these results show that FSP27, though being similar to perilipin, is an important lipid droplet protein with an independent function that is not compensated by other lipid droplet proteins such as perilipin.

Although FSP27 is different from perilipin, FSP27 is very similar to another lipid droplet protein ADRP. ADRP, similar to FSP27, targets to lipid droplets, and expression of ADRP in fibroblasts, COS, and hepatocytes has been shown to promote TG accumulation. The expression of ADRP increases early during adipogenesis, but declines in mature adipocytes as ADRP gets replaced by perilipin. In addition, both FSP27 and ADRP are ubiquitinated to target to proteasomal degradation (223). More interestingly, expression of ADRP increases in time dependent manner when adipocytes expressing C/EBP-α are treated with isoproterenol (229). This effect of isoproterenol on ADRP expression mirrors the effect of isoproterenol on FSP27. Moreover, the increased levels of ADRP after isoproterenol is associated with lipolysis dependent targeting of ADRP to lipid droplets as demonstrated by immunofluorescent microscopy (229). The increased levels of FSP27 after

isoproterenol treatment has also been shown to be associated with lipid droplets as shown biochemically as well as by immunofluorescent microscopy (258). However, isoproterenol mediated increases in ADRP expression has been shown to be independent of PKA activation because isoproterenol increases ADRP expression despite the presence of PKA inhibitor (229). Our results show that isoproterenol mediated increase in FSP27 is dependent on PKA activation as PKA depletion as well as PKA inhibitor abrogates the effect of isoproterenol on FSP27 expression. Previous studies also show that the up regulation of ADRP after isoproterenol treatment also required phosphorylation of perilipin (229). Whether phosphorylation of perilipin is required for the effect of isoproterenol on FSP27 remains to be investigated.

In addition to effects of lipid droplet proteins like FSP27 and perilipin, lipolysis ultimately depends on the enzymatic activity of lipases such as HSL and ATGL. Lipid droplet TG is hydrolyzed by ATGL into diacylglycerol that in turn is acted upon by HSL to release FFAs and glycerol (188, 192). It has been shown that HSL is required for catecholamine stimulated lipolysis (189, 280). The localization of HSL to lipid droplets could be perilipin phosphorylation independent in basal state and perilipin phosphorylation dependent in stimulated condition (219). However, the lipolytic effect of TNF-α has been considered to be due to its effect on various proteins and transcription factors that mediate changes in expression of multiple targets, and the requirement

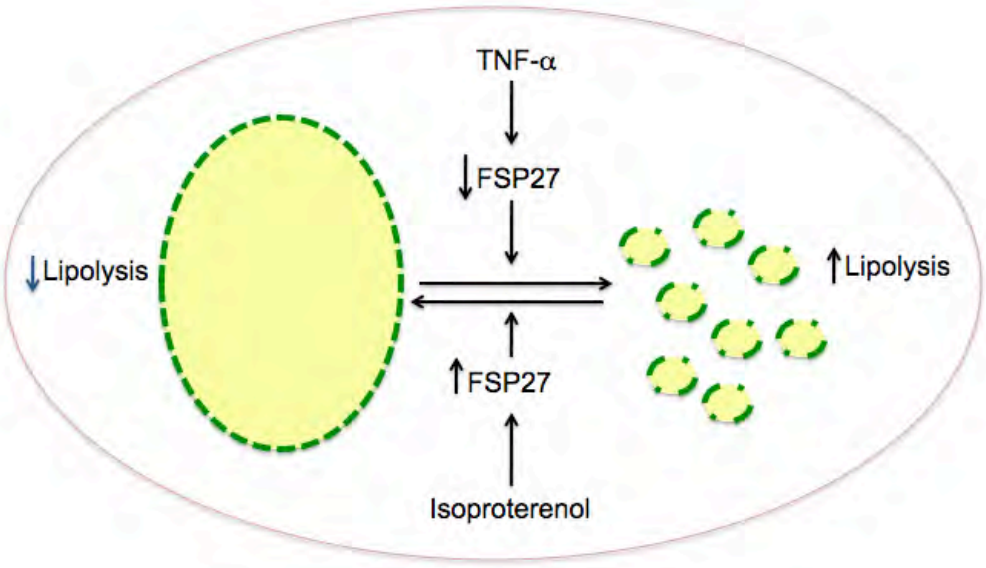


Fig. 2.14. Regulation of FSP27 by TNF-α and isoproterenol and their implications on lipolysis. TNF-α depletes FSP27 expression, that leads to formation of smaller lipid droplets from unilocular lipid droplet, and increase in lipolysis. Isoproterenol increases FSP27 expression, and the increase in FSP27 levels after isoproterenol treatment is required to protect lipid droplets from potent lipolytic activity of isoproterenol.

of specific lipases for its action has not been reported. Here we show that ATGL is required for TNF-α to stimulate lipolysis (Fig. 2.8 A). Furthermore, in the absence of lipase ATGL, depletion of FSP27 does not increase lipolysis. Thus, both FSP27 and TNF-α require ATGL to mediate their effects on lipolysis in adipocytes, similar to the actions of catecholamines. Important questions for future experiments are the molecular mode by which FSP27 mediates its effect on lipolysis and whether its mechanism of action involves direct or indirect engagement of the ATGL and HSL lipases, and/or inhbition of their lipase activities.

LIMITATIONS AND FUTURE PERSPECTIVES

All the experiments performed in this thesis work have been done in 3T3-L1 adipocytes. 3T3-L1 adipocytes in tissue culture are easy to handle, and are good model system to investigate metabolic parameters of adipocytes. These adipocytes have intact physiology of adipocytes *in vivo* such as the process of adipocyte differentiation, response to insulin, lipogenesis, and lipolysis. In addition, they make a model system in which hypothesis can be tested fairly quickly in physiologically relevant manner whereas investigating same questions in an animal model would take considerably more time. However, it needs to be acknowledged that adipocytes in tissue culture is deprived of the whole body milieu, and thus the results obtained in 3T3-L1

adipocytes need to be confirmed in *in vivo* model. We have observed that FSP27 expression increases during adipogenesis *in vitro* in 3T3-L1 adipocytes. Therefore, we may postulate that FSP27 will increase with obesity that is associated with increased fat mass. However, FSP27 expression decreases in *ob/ob* obese mice. This illustrates the importance of reconfirming the results obtained from *in vitro* model system in *in vivo* system also.

The data presented in this thesis indicate that the lipolytic actions of TNF-α and isoproterenol can be regulated through modulation of the FSP27 levels in adipocytes. It is evident from these studies that during TNF-α mediated lipolyis, depletion of FSP27 is a critical event, which if abrogated can impede the process of lipolysis also. On the other hand, up regulation of FSP27 in response to isoproterenol is a possible intrinsic mechanism to protect lipid droplets from the potent lipolytic effects of isoproterenol. However, it remains to be investigated in detail how FSP27 protects lipid droplets against lipolysis. It can be hypothesized that FSP27 covers lipid droplets to form a protective mechanical barrier to prevent the access of lipases to the TG core. It is also possible that FSP27 may inhibit the lipase activity of HSL and/or ATGL with or without direct interaction with these lipases.

Previous studies suggest that FSP27 promotes the formation of unilocular lipid droplets in adipocytes. Consistently, in my experiments, overexpression of FSP27 using an adenovirus resulted in formation of fewer and larger lipid droplets even in presence of TNF-α and isoproterenol, which are known to cause lipid droplet size diminution. This raises an interesting question whether FSP27 prevents the fragmentation of existing lipid droplets or promotes the fusion of smaller lipid droplets to form larger lipid droplets. It would be informative to use live cell imaging to study the characteristics and behaviour of lipid droplets in adipocytes containing or lacking FSP27.

Another important area for future investigations is to elucidate the mechanism of isoproterenol mediated FSP27 stabilization. Our results show that isoproterenol signals via canonical PKA pathway, and increases in cAMP levels to stabilize FSP27. However, FSP27 itself may not be phosphorylated suggesting involvement of other intermediate protein(s) that get(s) phosphorylated by PKA. It can be speculated that the intermediate protein(s), through direct or indirect mechanism, may target FSP27 to the lipid droplets harboring fatty acid reesterification, and the process of reesterification stabilizes FSP27. One of the possible candidates for such intermediate protein is the heat shock protein (HSP). Previous studies on HSPs have suggested their role in regulation of protein stability. In addition, HSPs can be possible targets for PKA mediated phophorylation because evidences

suggest that HSPs have several post-translational modifications including phophorylation. The unpublished data from our lab has identified HSP86 as a protein interacting with FSP27, and thus would be interesting to investigate if HSP86 gets phosphorylated by PKA, and plays a role in mediating effect of catecholamine on FSP27.

Although our data strongly show the involvement of FSP27 in lipolysis mediated by various stimuli in 3T3-L1 adipocytes, it would be preferable to repeat the results *in vivo.* It remains to be determined if transgenic mice engineered to express FSP27 specifically in adipose tissue will have decreased lipolysis in response to the lipolytic stimuli of TNF-α and isoproterenol.

Ultimately, it is imperative to demonstrate the observations from *in vitro* and rodent model are consistent in human also. Nonetheless, our study identifies modulation of FSP27 as an important mode by which TNF-α and isoproterenol regulate lipolysis in adipocytes.

Chapter III

DISCUSSION

In the present era of obesity and diabetes epidemic, studies researching the pathophysiology of insulin resistance in obesity and diabetes, and thereby contributing to find a cure for diabetes have paramount importance. One of the emerging hypotheses in the field of insulin signaling is the role of FFAs in pathogenesis of insulin resistance. Thus, there has been increasing interest in understanding the metabolism and regulation of free fatty acids. The key players in regulation of FFAs are the lipid droplet proteins that serve to store FFAs in the form of TGs in lipid droplets mainly in adipocytes, and various lipolytic stimuli that signal to hydrolyze stored TGs to release FFAs. This study examines the regulation of a novel lipid droplet protein, FSP27 by two major lipolytic stimuli, TNF-α and catecholamine, to modulate lipolysis in adipocytes.

Although FSP27 has been discovered in 1992 as an adipocyte-specific gene (262), FSP27 has emerged as lipid droplet protein only recently after our lab and others showed FSP27 to target to lipid droplets (233, 234, 249). The role of FSP27 in lipid metabolism is supported by studies that show the expression of FSP27 promotes lipid accumulation in oleic acid treated COS cells and preadipocytes whereas depletion of FSP27 using siRNA leads to degradaton of stored lipid leading to lipolysis and lipid droplet dispersion (233,

234). Moreover, FSP27 null mice are resistant to diet-induced obesity, and insulin resistance (234) suggesting the role of FSP27 in maintaining insulin sensitivity. Despite the growing attention to FSP27, the regulation of FSP27 and its role in lipid metabolism is still poorly understood.

Regulation of FSP27

This study demonstrates that FSP27 is a PPARγ responsive gene (231). PPARγ is considered to be the master regulator of adipogenesis, and is upregulated during adipogenesis. PPARγ controls the expression of numerous adipocyte-specific genes involved in lipid metabolism. Studies show that FSP27 is also upregulated during adipogenesis, and the increase in FSP27 expression mirrors the increase in PPARγ expression during the adipocyte differentiation process. Consistently, the expression of FSP27 increases in *ob/ob* mice upon treatment with PPARγ agonist rosiglitazone (231, 264). It has now been shown that PPARγ2 binds to PPARγ response element in FSP27 promoter, and drives the expression of FSP27 (246). FSP27 is also found to be PPARγ target gene in hepatocytes, and promotes hepatic steatosis in leptin-deficient mice (264). However, the mechanism of transcriptional control of FSP27, and the role of other transcription factor and co-factors remains to be investigated in detail.

In addition to the regulation of FSP27 at transcriptional level, FSP27 can also be regulated at translational or post-translational level. This study and recently published study show that FSP27 has an extremely short half-life of approximately 15 min (258). The half-life of FSP27 is short because it is ubiquitinated, and then degraded via proteasome. Regulation of FSP27 stability by ubiquitination is similar to the regulation of FSP27 isoform, CIDEA, and other lipid droplet proteins, perilipin and ADRP (209, 223, 238). Alteration of ubiquitin modification of FSP27 is one way how cellular FSP27 may be regulated. It is possible that the half-life of FSP27 can be much longer than 15 min when FSP27 is bound to lipid droplets or to proteins involved in fatty acid reesterification because Nian et. al. recently reported fatty-acid- re-esterification dependent stabilization of FSP27 in adipocytes after isoproterenol treatment. Another well-studied lipid droplet protein, perilipin, also has a short half-life when not bound to lipid droplet, but is stabilized to half-life of 40 hr when bound to lipid droplets (208). It remains open to exploration whether similar phenomenon exists to stabilize FSP27.

Depletion of FSP27 is critical for TNF-α to mediate lipolysis

TNF-α is an inflammatory cytokine that is shown to be elevated in adipocytes in obesity and inflammation (270) and also in circulation of diabetic NOD mice (269). Although TNF-α has myriad of effects that can lead to development of insulin resistance, one of the hypotheses is that the lipolytic effect of TNF-α

increases circulating FFAs that contribute to the development of insulin resistance. Prior studies show that TNF-α mediates lipolysis in adipocytes through multiple pathways including depletion of lipid droplet coat protein, perilipin, and inhibition of phosphodiesterase enzyme (174, 176). Here we show that depletion of FSP27 is a novel and critical pathway through which TNF-α mediates lipolysis in adipocytes.

TNF-α rapidly decreases FSP27 as observed by others and us (235) possibly due to the negative effect of TNF-α on PPARγ, the transcription factor that controls FSP27. However, the effect of TNF-α on FSP27 mRNA degradation cannot be excluded with present data. The rapid decrease in FSP27 protein after TNF-α treatment reflects the decrease in FSP27 mRNA. The effect of TNF-α on FSP27 protein may be primarily due to the effect on FSP27 transcription. Nevertheless, expression of FSP27 via adenovirus to overcome TNF-α mediated FSP27 depletion is able to protect against lipolytic effect of TNF-α in adipocytes. The adenovirus directed expression of FSP27 is also able to overcome the TNF-α mediated lipid droplet size diminution, consistent with our earlier results showing FSP27 promotes the formation of unilocular lipid droplets. TNF-α mediated lipolysis also involves perilipin, a lipid droplet protein that has similar role in lipolytic effect of TNF-α (174). However careful analysis of perilipin in our studies show that protective effect of FSP27 against lipolytic actions of TNF-α is independent of perilipin.

Although it has been observed previously that TNF-α causes lipid droplet size diminution, and increase in lipolysis, the temporal sequence of these events are not documented. This study show that TNF-α mediated rapid depletion of FSP27 is followed by lipid droplet size diminution that leads to increase in glycerol release. The depletion of FSP27 can be the key event that leads to lipid droplet dispersion, thereby increasing the total surface area of lipid droplets, and increase the access of lipases such as ATGL to the stored TGs. Once ATGL, the lipase necessary for TNF-α mediated lipolysis, gain access to the stored lipid, the enzymatic degradation of TGs releases FFAs and glycerol. Thus, it is observed that the increase in glycerol release after TNF-α treatment of adipocytes lag behind the TNF-α mediated FSP27 depletion, and lipid droplet dispersion.

The work presented here gives a new insight to the mechanism of TNF-α mediated lipolysis in adipocytes. These results suggest that FSP27 can be a potential target that can be modulated to overcome the TNF-α mediated lipolysis, and insulin resistance.

Isoproterenol mediated stabilization of FSP27

The other important stimuli that signal to lipolysis in adipocyte are catecholamines. Catecholamines, released in circulation during fasting,

stimulate β-adrenergic receptors in adipocytes causing robust lipolysis to release FFAs that act as fuel to other cell types. We report here that catecholamines paradoxically increase cellular FSP27 levels by delaying its proteasomal degradation.

The recently published study and our study (258) show that cellular FSP27 levels increase after isoproterenol treatment of adipocytes. Their study and ours show that isoproterenol mediated increase in FSP27 is due to decreased ubiquitination and degradation of FSP27. The work in this thesis further show that isoproterenol signals via canonical PKA pathway to stabilize FSP27, but the activation of PKA by isoproterenol does not phosphorylate FSP27 unlike perilipin that gets phosphorylated by PKA (181). Nian et. al. has reported that PKA mediated lipolysis releases FFAs that undergoes reesterification and the process of reesterification stabilizes FSP27. However, we show that isoproterenol stabilizes FSP27 even when lipolytic effect of isoproterenol is blunted by siRNA mediated ATGL knock-down.

We have also examined the functional implication of isoproterenol mediated FSP27 stabilization in adipocytes. Previous study has shown impaired isoproterenol-mediated lipolysis when FSP27 was depleted in 3T3-L1 adipocytes (272), however the data has been presented as a ratio of non-esterified fatty acids (NEFA) released in isoproterenol stimulated condition to

NEFA released in basal condition, and the decrease in the ratio seen in FSP27 silenced condition can be due to increased basal NEFA release due to FSP27 silencing. We show robust lipolysis in response to isoproterenol even when FSP27 is silenced using siRNA and additionally, these results show the rate of lipolysis after isoproterenol treatment of adipocytes is increased when the effect of isoproterenol on FSP27 stabilization is inhibited by knocking down FSP27 using siRNA. This suggests that FSP27 functions to protect against the catecholamine mediated lipolysis, and the stabilization of FSP27 by catecholamine could be a feedback mechanism to prevent excessive lipolysis after catecholamine treatment. Although there are conflicting results that show adenovirus directed expression of FSP27 to a higher level does not protect against the robust lipolytic effect of isoproterenol, it can be debated that isoproterenol treatment alone has already increased FSP27 to its maximal functional level, and thus additional FSP27 expression does not have any supplementary protective effect against isoproterenol mediated lipolysis.

Though adenovirus directed FSP27 expression does not diminish robust lipolytic effect of isoproterenol, the increased FSP27 levels interestingly protect against isoproterenol mediated dispersion of lipid droplets. Thus, this study confirms previous studies indicating isoproterenol mediated lipolysis

and lipid droplet dispersion are two distinct and different events, and the lipid droplet dispersion is not a prerequisite for lipolysis (183).

The work presented here taken together with the study done by Nian et. al. shows that FSP27 is a remarkably short-lived protein with a half-life of approximately 15 mins, and is ubiquitinated to target to proteasome. Isoproterenol delays the degradation of FSP27 and stabilizes FSP27. This thesis work furthers Nian et. al. study showing that isoproterenol mediated stabilization of FSP27 signals via canonical PKA pathway and increase in intracellular cAMP; but is independent of isoproterenol mediated lipolysis. The work presented here also attempts to tackle the physiological significance of change in FSP27 levels, and shows that the cytokine TNF-α depletes FSP27 to mediate lipolysis whereas isoproterenol stabilizes FSP27 to restrain its own potent lipolytic activity.

FSP27 is an emerging lipid droplet protein with an important function in lipid metabolism. The effect of FSP27 on inhibiting lipolysis and promoting TG accumulation in unilocular lipid droplets is not only seen *in vitro* and in rodent models, but also is observed in human adipocytes (257). In addition, it has been reported that a patient with homozygous mutation in FSP27/CIDEC has partial lipodystrophy, and insulin resistant diabetes (251). Our lab also has reported the decrease in FSP27/CIDEC in the insulin resistant obese patients

compared to the insulin sensitive obese ones (231). FSP27/CIDEC is one of the genes that is reported to be upregulated in patients in response to the therapeutic treatment of type II diabetes with rosiglitazone or metformin (247). Taken together, these evidences reinforce the importance of FSP27/CIDEC in lipid metabolism, and insulin sensitivity in humans consistent with the data obtained from *in vitro* and rodent model. Moreover, considering the protective effects of FSP27 against lipolysis, and insulin resistance, FSP27 could be a possible therapeutic target for treatment of type 2 diabetes mellitus.

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